# Universidade Nova de Lisboa

## Instituto de Higiene e Medicina Tropical


# The role of detoxification in the mosquito *Anopheles gambiae* response to *Plasmodium* infection


**Rute Castelo Félix**

Licenciada em Biologia pela Universidade de Évora


Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor no Ramo de Ciências Biomédicas, Especialidade em Parasitologia, realizada sob orientação científica do Prof. Dr. Henrique Silveira


**Orientador:** Prof. Dr. Henrique Silveira
Unidade de Parasitologia Médica
Instituto de Higiene e Medicina Tropical

**Co-Orientador:** Profª. Drª. Vera Ribeiro
Centro de Biomedicina Molecular e Estrutural
Universidade do Algarve

**Comissão Tutorial:** Dr. João Pinto
Unidade de Parasitologia Médica
Instituto de Higiene e Medicina Tropical



O trabalho foi financiado pela Fundação para a Ciência e Tecnologia, através da bolsa de doutoramento SFRH/BD/28024/2006 e dos projectos de investigação POCTI/SAU-IMI/59489/2004 e PTDC/SAUMII/102596/2008.


**JUNHO, 2011**

***Aos meus pais…***

# Acknowledgements

The present thesis would not have been possible without the important collaboration of several institutions and people to whom I would like to thank:

To the Instituto de Higiene e Medicina Tropical and to Centro de Malária e outras Doenças Tropicais for providing me with the necessary conditions to carry out my work. To the UEI Parasitologia Médica, where I developed my work, especially to Prof. Dr. Virgílio Estólio do Rosário for all the support as director of the unit during that time.

To the Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve for accepting me and giving me the necessary conditions to carry on my work.

To the Liverpool School of Tropical Medicine, for receiving me as a temporary student allowing me to perform the microarray analysis, which was fundamental to the beginning of this work.

To Fundação para a Ciência e Tecnologia (FCT) for the financial support provided by a PhD fellowship grant (SFRH/BD/28024/2006) and research funds from projects POCTI/SAU-IMI/59489/2004 and PTDC/SAUMII/102596/2008.

To Prof. Dr. Henrique Silveira, my supervisor, that encouraged and challenged me throughout the development of this thesis. His guidance, insights and discussions gave me the opportunity to grow as a researcher.

To Prof. Dr. Vera Marques, from CBME, University of Algarve, for accepting me as a student and for all the good advices during the development of this work.

To Dr. João Pinto, for accepting to be a member of my tutorial commission and for accompanying this work.

To Hilary Ranson, from the Liverpool School of Tropical Medicine, for welcoming me to her lab, for all her support and help in discussing our results. And to Pie Müller, for teaching me all about microarray analysis.

To my present and former colleagues in IHMT, for their friendship, help, suggestions and for always being available. To Ana Catarina Alves for her friendship and for maintaining the *A. gambiae* insectary and to Dinora Ferreira, for her friendship and contagiously daily joy.

A special thanks to Patrícia Machado and to Cristina Mendes for all their support, for the late talks in the lab, for listening and always being there whenever I needed, for brighten life in the lab, making my life so much easier.

To my colleagues Jorge Correia and Ana Ribeiro, for all the support in the lab, for the healthy work related discussions and for always having time for a coffee.

To all my friends for the support and friendship during these years, lending a sympathetic ear instead of running away, cheering me up in the most difficult moments.

To my parents and sister for all the support, strength, motivation, practical advice, love and confidence entrusted in me.

To João Tiago Tavares, for all the schemes, illustrations and opinions about my work and for all the unconditional support and love he gave me...

# Resumo

## O papel da destoxificação na resposta do mosquito *Anopheles gambiae* à infecção por *Plasmodium*

**Rute C. Félix**

**PALAVRAS-CHAVE:** Malária, mosquito vector, *Anopheles gambiae*, parasita, *Plasmodium berghei*, infecção, enzimas de detoxificação, citocromos P450, tubulinas

A malária, uma das doenças mais devastadoras que ocorrem em África é causada por um parasita do género *Plasmodium* e é transmitida aos humanos por mosquitos vectores do género *Anopheles* durante a refeição de sangue. Apesar da resposta do mosquito à infecção por *Plasmodium* ter vindo a ser intensamente estudada nos últimos anos, as interacções entre o mosquito vector e o parasita são muito complexas e, estão longe de serem completamente compreendidas. Este estudo tem como objectivo principal contribuir para o conhecimento da resposta do mosquito à infecção por *Plasmodium*, focando-se no papel das enzimas de detoxificação. Para atingir este objectivo realizou-se uma análise transcriptómica com microarrays, com o intuito de identificar alterações de transcrição de enzimas de detoxificação no mosquito *Anopheles gambiae* em resposta à infecção por *Plasmodium*. Esta análise permitiu identificar alterações na expressão de 254 genes de destoxificação no estômago e corpo gordo de *A. gambiae* durante a invasão do intestino médio pelos oocinetos e durante a libertação dos esporozoítos do oocisto. Os resultados mostraram que a invasão do intestino médio pelos oocinetos causou alterações num maior número de genes em ambos os tecidos estudados, sendo o intestino médio do mosquito o tecido mais afectado nas duas fases da infecção do parasita. De todos os genes de destoxificação com expressão alterada, as tubulinas e os citocromos P450 destacaram-se e foram escolhidos para continuar o estudo. As tubulinas foram seleccionadas porque estão associadas à invasão do epitélio do intestino médio e a sua função na resposta à invasão do *Plasmodium* ainda não está bem definida. Os citocromos P450 foram seleccionados porque já foram descritos como tendo a expressão alterada em resposta ao *Plasmodium* e a outras infecções. Para identificar e caracterizar o papel das tubulinas durante a infecção pelo parasita e a sua possível associação com os citocromos P450 foi utilizado o silenciamento génico por RNA de interferência e a injecção de inibidores químicos de tubulinas. O silenciamento e co-silenciamento das tubulinas causaram um aumento da taxa e intensidade da infecção. No entanto, apesar de o aumento ser consistente não foi significativo. Por outro lado, a injecção de paclitaxel, um inibidor de tubulinas, aumentou significativamente a taxa e intensidade da infecção, fortalecendo a hipótese do envolvimento das tubulinas na resposta à infecção por *Plasmodium*. Este trabalho também mostrou que o co-silenciamento da *tubulina A* e *tubulina B* e a injecção do

inibidor de tubulinas colchicine causam alterações significativas na expressão da *CYP6Z2*, sendo este proposto como um possível elo de ligação entre as tubulinas e os citocromos P450. Finalmente, uma análise comparativa foi realizada para estudar as regiões promotoras dos citocromos P450: *CYP6M2* e o *CYP6Z1*. Este estudo obteve novos dados sobre compostos que activam estes citocromos e quais os possíveis factores de transcrição envolvidos. Dos diferentes estímulos utilizados, a exposição a insecticidas e a bactérias foram os que mais afectaram estes citocromos. O conjunto total das diferentes abordagens utilizadas neste trabalho contribuiu para aumentar o conhecimento do papel das enzimas de destoxificação durante a passagem do parasita da malária pelo mosquito vector.

# Abstract


## The role of detoxification in the mosquito *Anopheles gambiae* response to *Plasmodium* infection

**Rute C. Félix**

**KEYWORDS:** Malaria, mosquito vector, parasite, *Anopheles gambiae*, *Plasmodium berghei*, infection, detoxification enzymes, P450 cytochromes, tubulins

Malaria, one of the most devastating diseases in Africa, is caused by protozoan parasites of the genus *Plasmodium* and is transmitted to humans by mosquito vectors of the genus *Anopheles* during their blood meal. Although the mosquito responses to *Plasmodium* infection have been intensely studied in the last years, the interactions between the mosquito vector and the malaria parasite are extremely complex and are far from being totally understood. This study aims to contribute for the knowledge of the complex mosquito response to *Plasmodium*, focusing on the role of detoxification enzymes. To achieve this, a microarray-based transcriptional profiling was performed to identify transcriptional changes in detoxification enzymes in the mosquito *Anopheles gambiae* in response to *Plasmodium* infection. This analysis allowed a comprehensive knowledge of the transcription profile of 254 detoxification genes in the midgut and fat body of *A. gambiae* during the ookinete invasion of the midgut epithelium and during the sporozoites release from the oocysts. The results showed that the ookinete invasion of the midgut epithelium caused a higher number of genes to be differentially expressed in both tissues, being the mosquito midgut the most affected tissue in both phases of the parasite invasion. From all the relevant differentially expressed detoxification genes, tubulins and P450 cytochromes stood out and were chosen as targets for further study. Tubulins were selected because their function in the response to *Plasmodium* invasion is not well defined yet. P450 cytochromes were selected because they were described to be differentially expressed in response to *Plasmodium* as well as to other infections. A reverse genetic analysis by RNA silencing and injection of tubulin inhibitors was used to identify and characterize the role of tubulins during the development of parasite infection and their possible association with P450 cytochromes. The silencing and co-silencing of tubulins caused an increase in the infection rate and intensity. Nevertheless, although this increase was consistent it was not significant. On the other hand the injection of paclitaxel, a tubulin inhibitor, significantly increased the infection rate and intensity, further suggesting the involvement of tubulins in the response to *Plasmodium* infection. This work also showed that the co-silencing of *tubulinA* and *tubulinB* and the injection of tubulin inhibitor colchicine causes a significant change on the expression of *CYP6Z2*, which has been identified as the possible link of connection between tubulins and P450 cytochromes. Finally, a comparative approach was made to study the

promoter regions of P450 cytochromes: *CYP6M2* and *CYP6Z1*. This study provided new data on compounds that activate these P450 cytochromes and the putative transcription factors involved. Of the different challenges used, insecticide exposure and bacterial infection were the ones that affected these P450s the most. Altogether, this set of approaches contributed to further understand the role of the detoxification enzymes during the malaria parasite life cycle stages inside the mosquito vector.

# Abbreviations

| | |
|---|---|
| A. | *Anopheles* |
| AP-1 | Activator protein 1 |
| B2M | Beta-2-microglobulin |
| bp | Base pairs |
| C/EBP | CCAAT-enhancer-binding proteins |
| cDNA | complementary deoxyribonucleic acid |
| CPR | Cytochrome P450 reductase |
| CYP | Cytochrome P450 |
| DDT | Dichlorodiphenyltrichloroethane |
| DEPC | Diethylpyrocarbonate |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dsRNA | Double-stranded ribonucleic acid |
| F | Fisher's Exact test |
| FAD | Flavin adenine dinucleotide |
| FMN | Flavin mononucleotide |
| GATA | GATA transcription factors |
| GST | Glutathione S-transferase |
| KD | Knock down |
| MW | Mann-Whitney test |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-$_K$B | Nuclear factor kB |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |

| | |
|---|---|
| P. | *Plasmodium* |
| P450s | P450 cytochromes |
| PBS | Phosphate- buffered saline |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RNOS | Reactive nitrogen oxide species |
| RPS7 | Ribosomal protein S7 |
| RT-qPCR | Quantitative Real time – Polymerase chain reaction |
| SEM | Standard error of the mean |
| SOD | Superoxide dismutase |
| *tuba* | tubulinA |
| *tubB* | tubulinB |
| WHO | World Health Organization |

**Nucleotide Bases**

| | |
|---|---|
| A | Adenine |
| C | Cytosine |
| G | Guanine |
| T | Thymine |
| M | A or C |
| N | Any nucleotide (A, C, G or T) |
| R | Purine (A or G) |
| Y | Pyrimidine (C or T) |
| K | G or T |
| W | A or T |
| D | A, G or T |

# Table of Contents

# List of Figures

**Chapter 5 - Promoter analysis of three P450 cytochromes in *Anopheles gambiae***

# List of Tables

# Chapter 1 – Introduction

## Malaria

Malaria is a severe mosquito-borne disease, which persists today as one of the most widespread and devastating parasitic infections affecting the human population. There were an estimated 225 million cases of malaria in 2009 that accounted for approximately 781000 deaths, most of them of children under five years old living in sub-Saharan Africa (WHO, 2010).

Malaria is caused by protozoan parasites from the genus *Plasmodium*, which are transmitted to humans when female mosquitoes of the genus *Anopheles* feed on human blood.

## Parasite

*Plasmodium* spp. are obligate parasites. They have a complex life cycle involving two hosts: a vertebrate host and a mosquito vector. In the mosquito vector parasites develop their sexual life cycle (sporogonic cycle) and in the vertebrate host they complete their asexual life cycle, which can be divided in hepatic and erythrocytic, the latter being responsible for the malaria symptoms. *Plasmodium* life cycle (adapted from (Knell, 1991)) is shown in detail in Figure 1.

The sporogonic cycle starts when *Plasmodium* enters the midgut with the blood meal. Gametocytes undergo differentiation into gametes and fertilization occurs forming diploid zygotes. The zygotes become motile and develop into ookinetes. Approximately 24 hours after the blood meal the motile ookinetes leave the blood bolus invading the midgut epithelium and settle under the basal lamina forming vegetative oocysts. During the oocysts maturation, the parasites undergo a meiotic cycle followed by several rounds of mitosis to form haploid sporozoites. After maturation the oocysts rupture releasing the sporozoites into the hemolymph. The sporozoites travel through the mosquito hemacoel until they reach the salivary glands, which they invade through the salivary ducts. During a subsequent blood meal, the sporozoites are injected along with saliva into the host, starting the vertebrate host phase. The injected sporozoites rapidly infect the liver, where they multiply forming a new invasive form, the merozoite. The merozoites are then released into the blood stream and invade red blood cells. Here they

mature into schizonts and undergo a series of divisions, forming new invasive merozoites, initiating new cycles of erythrocyte invasion, maturation and rupture which causes the symptoms of the malaria illness. Some merozoites differentiate into gametocytes that are taken up by the mosquito in a blood meal initiating a new cycle (Knell, 1991).

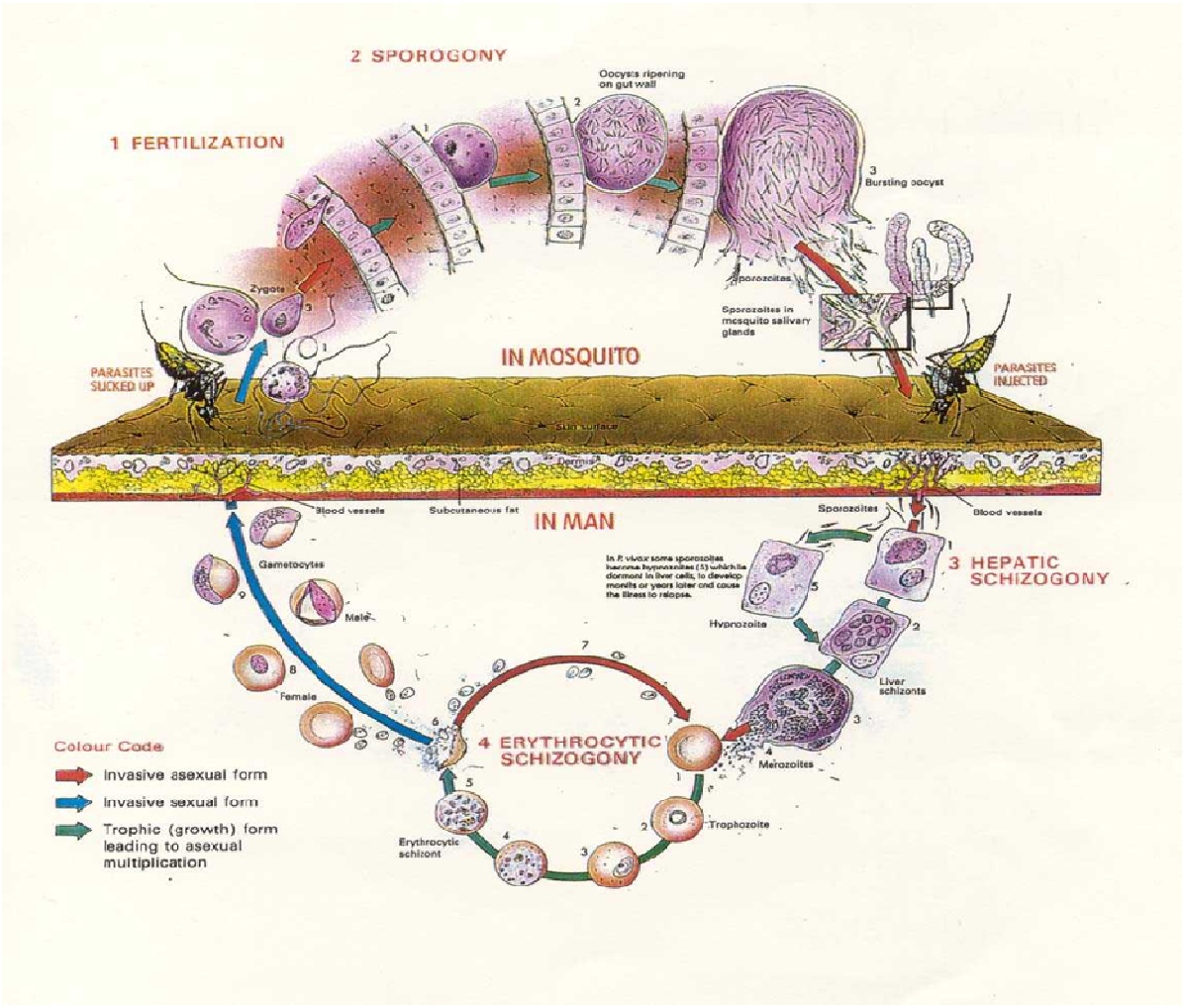



**Figure 1 –** Life cycle of *Plasmodium* (adapted from (Knell, 1991)).

## Malaria, an ongoing problem

In all tropical and subtropical parts of the world, malaria maintains a high prevalence. The hot and humid weather, the poor health and sanitary system existent in these parts of the globe and the interruption/ceasing of control programs contribute to the success of the development and reproduction of the mosquito vector. This, together with i) the absence of an efficient protective vaccine, ii) the rapid spread of parasite resistance to anti-malarial drugs and iii) the increasing resistance to insecticides of the mosquito vector, perpetuates the occurrence and, in some countries, is accountable for the increase of malaria cases, highlighting the fragility of malaria control and the need to maintain/increase control programs and the implementation of novel and more effective approaches.

The malaria parasite depends on its development inside the mosquito to infect a human host. In this context, there are two efficient strategies that are commonly used to reduce malaria transmission: 1) to avoid the contact between mosquitoes and humans by using insecticide impregnated bednets and repellents, and 2) to lower mosquito numbers by insecticides and eliminating potential breeding sites. However, the increase of insecticide resistance is increasingly threatening the efficacy of these control strategies. The major resistance mechanisms in insects can be divided in two groups: target site resistance and metabolic resistance (Hemingway et al., 2004), and are reviewed in chapter 2. A potential new approach to block malaria transmission is the interruption of the parasite development in the vector by targeting essential mechanisms for the development of the parasite. The development of such a strategy requires a detailed knowledge of the biology of the mosquito and the parasite, as well as the nature of their complex interactions, that define an efficient parasite–vector system. Thus understanding and identifying key molecules that are vital for the successful development and transmission of the malaria parasite would have a great impact in reducing malaria transmission.

## *Anopheles gambiae* mosquito

*Anopheles gambiae* is the major African vector for transmission of malaria. It became a suitable model for the study of innate immunity as *A. gambiae* mounts efficient local and systemic immune responses against *Plasmodium* infection and its genome has been sequenced (Holt et al., 2002) and since then regularly annotated.

The combination *A. gambiae* and the rodent parasite *Plasmodium berghei* is one of the most used model systems for laboratory assays of parasite infections. The existence of poor or incompatible vector-parasite combinations suggests that specific molecular and cellular interactions are essential for a vector-parasite system to become established and subsequently co-evolve (Alavi et al., 2003). Of all the interactions between the mosquito and the parasite, the ones that occur during the three main bottlenecks (where the parasite numbers are largely reduced and its development can be disrupted) are the most important (Blandin and Levashina, 2004). The extreme parasite losses are attributed to efficient mosquito immune responses, which can completely block parasite development.

## Detoxification enzymes

*Anopheles gambiae* becomes infected with the malaria parasite by taking a blood meal. The blood meal alone triggers the transcriptional regulation of several mosquito genes required to blood digestion causing metabolic changes which induce a state of oxidative stress (Vlachou et al., 2005, Kumar et al., 2003). A *Plasmodium* infected blood meal induces the production of reactive oxygen and nitrogen species to contain parasite infection (Molina-Cruz et al., 2008, Kumar et al., 2003), further increasing the oxidative stress caused by blood digestion (Molina-Cruz et al., 2008). Inside the mosquito high levels of oxidative stress could become toxic so it needs to be decreased/eliminated by detoxification enzymes. There are three main detoxification families in *A. gambiae*: glutathione-S-transferases (GST), carboxylesterases and P450 cytochromes (Ranson et al., 2002). P450 cytochromes are a superfamily of monooxygenases that have several functional roles, including growth, development and feeding. They are involved in the metabolism and detoxification of both exogenous compounds like insecticides and

endogenous compounds like steroids (Feyereisen, 1999, Scott, 1999). An actualized description about insect P450 cytochromes and their putative role in *A. gambiae* is reviewed in chapter 2.

The exact role of detoxification enzymes in the mosquito response to *Plasmodium* infection is not well known. The impact of *Plasmodium* infection on the expression of detoxification genes in the midgut and fat body of *A. gambiae* mosquitoes is described in chapter 3. Two time points, decisive for parasite invasion: day 1 post-infection (p.i.) (when the ookinete is traversing the midgut epithelium) and day 11 p.i. (when the sporozoites are release to the hemolymph) were analysed.

## Cytoskeleton genes

During *Plasmodium* infection differential regulation of cytoskeleton genes has been observed (Vlachou et al., 2005, Marinotti et al., 2005, Abrantes et al., 2008). The cytoskeleton rearrangement was described as a protective mechanism during ookinete invasion of the midgut in *A. gambiae* (Vlachou et al., 2005). Tubulin disarray could be responsible for the regulation of several P450 cytochromes, causing their suppression or induction during the mosquito response to parasite invasion. Similar to the association described in mammals (Dvorak et al., 2005). An attempt to clarify the role of tubulins on parasite invasion and their interplay with P450 cytochromes is described in chapter 4.

## P450 promoter regions

Previous studies suggested the involvement of P450 cytochromes in the response to microbial, parasitic and insecticide challenges (David et al., 2005, Djouaka et al., 2008, Dong et al., 2009, Pinto et al., 2009). However, we are still unable to specify the exact role of the P450 cytochromes in these responses and to which challenges they responded. The study of promoter regions and the identification of specific transcription factors binding sites will allow the association of these factors with the challenge they respond to. Chapter 5 describes a comparative approach to identify specific

transcription factor binding sites within three P450 promoter regions previously associated with infection and insecticide resistance.

# Aims of this thesis

Malaria is not a problem from the past. Even today the number of new malaria cases is rising in some of the poorest tropical and subtropical countries. It is increasingly important to take efficient measures and to find new strategies to control malaria transmission. For this it is essential to understand the biology of the parasite and the mosquito and specially the interactions between them. Thus, the complete knowledge of the mosquito immune response to the invading parasite is essential. The *A. gambiae* innate immunity has been extensively studied over the last years, and yet there are still several unknown mechanisms of protection.

The present work aimed to study the role of detoxification genes of *A. gambiae* in response to *P. berghei*. As there are many detoxification enzymes in the genome of *A. gambiae* and they have a great variety of functions, the study focused on the role of P450 cytochromes.

## Specific objectives

**1 –** Identification of the *A. gambiae* mosquitoes detoxification enzymes, which are differentially expressed during *P. berghei* invasion of midgut epithelium and sporozoite egress from the oocyst.

**2 –** Characterization of the role of *A. gambiae* tubulins in the response to *Plasmodium* infection and its interaction with the regulation of P450 cytochromes.

**3 –** Identification of putative transcription factors binding sites within the promoter regions of P450 cytochromes that are differentially regulated during infection.

## References

Abrantes, P., Dimopoulos, G., Grosso, A. R., Do, R. V. & Silveira, H. 2008. Chloroquine mediated modulation of Anopheles gambiae gene expression. *PLoS.One.,* 3**,** e2587.

Alavi, Y., Arai, M., Mendoza, J., Tufet-Bayona, M., Sinha, R., Fowler, K., Billker, O., Franke-Fayard, B., Janse, C. J., Waters, A. & Sinden, R. E. 2003. The dynamics of interactions between Plasmodium and the mosquito: a study of the infectivity of Plasmodium berghei and Plasmodium gallinaceum, and their transmission by Anopheles stephensi, Anopheles gambiae and Aedes aegypti. *Int.J.Parasitol.,* 33**,** 933-943.

Blandin, S. & Levashina, E. A. 2004. Mosquito immune responses against malaria parasites. *Curr.Opin.Immunol.,* 16**,** 16-20.

David, J. P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P. M., Louis, C., Hemingway, J. & Ranson, H. 2005. The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proc.Natl.Acad.Sci.U.S.A,* 102**,** 4080-4084.

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C., Ranson, H., Hemingway, J. & Strode, C. 2008. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. *BMC.Genomics,* 9**,** 538.

Dong, Y., Manfredini, F. & Dimopoulos, G. 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS.Pathog.,* 5**,** e1000423.

Dvorak, Z., Ulrichova, J. & Modriansky, M. 2005. Role of microtubules network in CYP genes expression. *Curr.Drug Metab,* 6**,** 545-552.

Feyereisen, R. 1999. Insect P450 enzymes. *Annu.Rev.Entomol.,* 44**,** 507-533.

Hemingway, J., Hawkes, N. J., Mccarroll, L. & Ranson, H. 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem.Mol.Biol.,* 34**,** 653-665.

Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M., Wides, R., Salzberg, S. L., Loftus, B., Yandell, M., Majoros, W. H., Rusch, D. B., Lai, Z., Kraft, C. L., Abril, J. F., Anthouard, V., Arensburger, P., Atkinson, P. W., Baden, H., De, B. V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chaturverdi, K., Christophides, G. K., Chrystal, M. A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C. A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M. E., Hladun, S. L., Hogan, J. R., Hong, Y. S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J. J., Lobo, N. F., Lopez, J. R., Malek, J. A., Mcintosh, T. C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S. D., O'brochta, D. A., Pfannkoch, C., Qi, R., Regier, M. A., Remington, K., Shao, H., Sharakhova, M. V., Sitter, C. D., Shetty, J., Smith, T. J., Strong, R., Sun, J., Thomasova, D., Ton, L. Q., Topalis, P., Tu, Z., Unger, M. F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K. J., Wortman, J. R., Wu, M., Yao, A., Zdobnov, E. M., Zhang, H., Zhao, Q., et al. 2002. The genome sequence of the malaria mosquito Anopheles gambiae. *Science,* 298**,** 129-149.

Knell, A. J. 1991. *Malaria: A publication of the tropical programme of the Wellcome Trust,* Oxford, Oxford University Press

Kumar, S., Christophides, G. K., Cantera, R., Charles, B., Han, Y. S., Meister, S., Dimopoulos, G., Kafatos, F. C. & Barillas-Mury, C. 2003. The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae. *Proc.Natl.Acad.Sci.U.S.A,* 100**,** 14139-14144.

Marinotti, O., Nguyen, Q. K., Calvo, E., James, A. A. & Ribeiro, J. M. 2005. Microarray analysis of genes showing variable expression following a blood meal in Anopheles gambiae. *Insect Mol.Biol.,* 14**,** 365-373.

Molina-Cruz, A., Dejong, R. J., Charles, B., Gupta, L., Kumar, S., Jaramillo-Gutierrez, G. & Barillas-Mury, C. 2008. Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium. *J.Biol.Chem.,* 283**,** 3217-3223.

Pinto, S. B., Lombardo, F., Koutsos, A. C., Waterhouse, R. M., Mckay, K., An, C., Ramakrishnan, C., Kafatos, F. C. & Michel, K. 2009. Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. *Proc.Natl.Acad.Sci.U.S.A,* 106**,** 21270-21275.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M. V., Unger, M. F., Collins, F. H. & Feyereisen, R. 2002. Evolution of supergene families associated with insecticide resistance. *Science,* 298**,** 179-181.

Scott, J. G. 1999. Cytochromes P450 and insecticide resistance. *Insect Biochem.Mol.Biol.,* 29**,** 757-777.

Vlachou, D., Schlegelmilch, T., Christophides, G. K. & Kafatos, F. C. 2005. Functional genomic analysis of midgut epithelial responses in Anopheles during Plasmodium invasion. *Curr.Biol.,* 15**,** 1185-1195.

Who 2010. World Malaria Report 2010. Geneve, Switzerland: World Health Organization.

# Chapter 2 - The role of *Anopheles gambiae* P450 cytochrome in insecticide resistance and infection

**This chapter was accepted for publication as a book chapter:**

Félix, R.C. and Silveira, H. The role of *Anopheles gambiae* P450 cytochrome in insecticide resistance and infection. Insecticides / Book 1. Editor Dr. Farzana Perveen. InTech - Open Access Publisher. ISBN 978-953-307-895-3.

## Introduction

*Anopheles gambiae* is the major vector of malaria transmission in sub-Saharan Africa where the disease is responsible for the highest morbidity and mortality worldwide. Malaria, nowadays, is still a major burden causing the death of nearly one million people each year, mostly children under the age of five, and affecting those living in the poorest countries (World Health Organization [WHO], 2010).

Currently, the major obstacles to malaria eradication are the absence of a protective vaccine, the spread of parasite resistance to anti-malarial drugs and the mosquito resistance to insecticides. Controlling mosquito vectors is fundamental to reduce mosquito-borne diseases. In fact, it has been one of the most used and effective method to prevent malaria, namely trough insecticides spraying and impregnated bed nets. These methods are highly dependent on a single class of insecticides, the pyrethroids, which are the most frequently used compounds for indoor residual spraying, and the only insecticide class used for insecticide treated nets (WHO, 2010). The extensive use of a single class of insecticides further increases the risk of mosquitoes developing resistance, which could rapidly lead to a major public health problem mainly in sub-Saharan countries where insecticidal vector control is being used widely (WHO, 2010). Strategies to control malaria are still not enough to totally eliminate malaria transmission, having yet to overcome several difficulties as the development of parasite drug resistance and mosquito-vector insecticide resistance (Yassine & Osta, 2010). Unfortunately the emergence of mosquito populations capable of withstanding insecticide exposure is threatening the efficiency of these control measures.

## Insecticide resistance

Resistance has been defined as 'the inherited ability of a strain of some organisms to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species' (Scott, 1999). The evolution of insecticide-resistant mosquito strains is an increasing problem and one of the major obstacles for the control of medical and agricultural arthropod pests. Therefore, a better understanding of its

genetic and biological basis is critical. Insecticide resistance can also lead to outbreaks of human diseases when vectors cannot be controlled. Hence, the elucidation of resistance mechanisms is extremely important for the development of tools to monitor resistance in populations, thereby contributing to mosquito control programs. Although the mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance *foci* (Brogdon & McAllister, 1998).

The main forms of resistance mechanisms can be divided in two groups: target site resistance, which occurs when the insecticide no longer binds to its target, and metabolic resistance, which occurs when enhanced levels of modified activities of detoxification enzymes prevent the insecticide from reaching its site of action. Alone or in combination these mechanisms confer resistance, sometimes at high levels, to all classes of insecticides.

***Target site resistance***

Target site resistance is based on alterations of amino acids in the site of action where the insecticide is supposed to bind, causing the insecticide to be less effective or ineffective at all. Knock down resistance (*Kdr*) occurs due to a single or multiple substitutions in the sodium channel (Martinez-Torres et al., 1998, Ranson et al., 2000a) and alteration in acetylcholinesterase results in decreased sensitivity to insecticides (Mutero et al., 1994). Insecticide resistance has been reported from many insects including *A. gambiae* that showed the presence of insensitive acetylcholinesterase in two different populations that were resistant to carbosulfan, a carbamate insecticide (N'Guessan et al., 2003). Mutations at a single codon in the *Rdl* (resistance to dieldrin) gene have been documented in all dieldrin-resistant insects, and results in conferring both insensitivity to the insecticide and also a decrease rate of desensitisation (ffrench-Constant et al., 1998). However, in *A. gambiae* this type of resistance mechanisms was not described until now. Those are examples of target site resistance that is not the object of the present work and therefore is reviewed elsewhere.

### *Metabolic resistance*

Metabolic resistance usually involves over-expression of enzymes capable of detoxifying insecticides or alterations in the amino acids within these enzymes causing alterations in the levels or activities of detoxification proteins. There are three major enzyme families involved in this type of resistance, GST, carboxylesterases and P450 cytochromes. Carboxylesterases are mainly involved in organophosphate and carbamate and to a lesser extent in pyrethroid resistance, while P450 cytochromes are mainly involved in the metabolism of pyrethoids and to a lesser extent, detoxification of organophosphates and carbamates (Hemingway & Ranson, 2000). Glutathione S-transferases are involved in the detoxification of a wide range of xenobiotics, including the organochloride insecticide DDT (Enayati et al., 2005). In *A. gambiae* metabolic resistance to insecticides can be conferred by elevation in the activity of these three classes of detoxifying enzymes.

The over-expression of carboxylesterases as an evolutionary response to organophosphorus and carbamate insecticide selection pressure has been reported in several insects, including mosquitoes (Newcomb et al., 1997; Vulule et al., 1999; Zhu et al., 1999). Organophosphorus and carbamate inhibit B esterases by rapid esterification of the serine residue in the active site, usually followed by a slow hydrolysis of the new ester bond. Therefore, these insecticides can be considered as inhibitors of esterases, because they are poor substrates which have a high affinity for these enzymes (Hemingway & Karunaratne, 1998). Carboxylesterases in large amounts causes resistance as the insecticides are rapidly sequestered, even before reaching the target-site acetylcholinesterase (Hemingway & Karunaratne, 1998). There are many reports of over expression of carboxylesterases in insecticide resistant mosquitoes including *A. gambiae*, where enhanced production of carboxylesterases was observed in permethrin-resistant mosquitoes (Vulule et al., 1999).

Glutathione S-transferases are a major class of detoxification enzymes that possess a wide range of substrates specificities (Enayati et al., 2005). Elevated GST activity has been implicated in resistance to several classes of insecticides (Ranson et al., 2001). Higher enzyme activity is usually due to an increase in the amount of one or more enzymes, either as a result of gene amplification or more commonly through increases

in transcriptional rate, rather than qualitative changes in individual enzymes (Hemingway et al., 2004). The primary function of GSTs is the detoxification of both endogenous and xenobiotic compounds either directly or by catalysing the secondary metabolism of a vast array of compounds oxidised by P450 cytochromes (Wilce & Parker, 1994). GST enzymes metabolise insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione to produce water soluble metabolites that are more readily excreted (Wilce & Parker, 1994). They also contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati et al., 2005). In *A. gambiae* elevated GST levels were shown to be associated with DDT resistance (Ranson et al., 2001). Furthermore genetic mapping of the major *loci* conferring DDT resistance in *A. gambiae* implicate both *cis*- and *trans*-acting factors in the overexpression of GSTs (Ranson et al., 2000b). GSTs in *A. gambiae* were also over expressed in a DDT-resistant strain, but only one *GSTE2-2* was able to metabolise DDT (Ortelli et al., 2003).

P450 cytochromes are a complex family of enzymes that are involved in the metabolism of xenobiotics and have a role in the endogenous metabolism. P450 cytochromes mediated resistance is probably the most frequent type of insecticide resistance. They are involved in the metabolism of virtually all insecticides, leading to activation of the molecule in the case of organophosphorus insecticides, or more generally to detoxification (Scott & Wen, 2001). In most cases where a link between insecticide resistance and elevated P450 activity has been shown, the P450 cytochrome belongs to the *CYP6* family (Nikou et al, 2003; Djouaka et al. 2008; Müller et al, 2007; McLaughlin et al., 2008). Although being difficult the identification of the specific P450 cytochrome associated with resistance, several P450 cytochromes were already isolated from insecticide resistant strains (Dunkov, et al., 1997; Kasai & Scott, 2000, Sabourault et al., 2001).

## Insect P450 cytochromes

P450 cytochromes are hemoproteins which act as terminal oxidases in monooxygenase systems. P450 cytochromes, whose name originated on its characteristic absorbance peak at 450 nm that appears when these enzymes are reduced and saturated with carbon-monoxide, constitute one of the oldest and largest super families of enzymes being found in almost all living organisms. In the literature, P450 enzymes are known by several names: cytochromes P450 monooxigenases, mixed functions oxidases, microsomal oxidases and heme thiolate proteins.

Insect P450s play a critical role in the metabolism of a wide variety of endogenous and exogenous compounds such as steroids, fatty acids and a wide range of xenobiotics and have also been implicated in vital processes like growth, development, feeding, reproduction, insecticide resistance and tolerance to plants toxins (Feyereisen, 1999; Scott et al., 1998; Scott, 1999). P450 cytochromes are also intimately involved in the synthesis and degradation of insect hormones and pheromones, including 20-hydroxyecdysone and juvenile hormone (Feyereisen, 1999).

### *Nomenclature*

To distinguish one of these cytochromes among all the P450s, a standardized nomenclature system was implemented (Nelson et al., 1996; Nebert et al., 1991). Each P450 is named with CYP, followed by an Arabical number for the gene family, a letter for the sub-family and another Arabical number for the gene. Cytochromes P450s with share more then 40% of the amino acids are usually grouped into the same family and members with >55% of the amino acids identical are normally grouped in the same sub-family. However, there are exceptions to these rules (Nelson et al., 1996). As it is based on amino acid similarities, no information regarding the function of each P450 should be assumed from its name.

### *Structure*

P450s can be divided into classes depending on how electrons from NAD(P)H are delivered to the catalytic site. Class I P450s are found in eukaryotes and are associated

with mitochondrial membranes. This class of enzymes requires both a FAD-containing reductase and an iron sulphur redoxin, and catalyzes several steps in the biosynthesis of steroid. Class II enzymes are the most common in eukaryotes and are found in the endoplasmic reticulum. These enzymes only require an FAD/FMN-containing P450 reductase for transfer of electrons. Their functions are extremely diverse and, in eukaryotes, include aspects of the biosynthesis and catabolism of signalling molecules and steroid hormones (Feyereisen, 1999). Class III enzymes are self-sufficient and require no electron donor. They are involved in the synthesis of signalling molecules. Finally, class IV enzymes receive electrons directly from NAD(P)H. Class I and II P450s from all organisms participate in the detoxification or sometimes the activation of xenobiotics and class III and IV enzymes are considered remains of the ancestral forms of P450s involved in detoxification of damaging activated oxygen species (Werck-Reichhart & Feyereisen, 2000).

Most P450s are approximately 500 amino acids long. The core of these proteins is formed by a four-helix bundle, two sets of $\beta$ sheets, two helices and a coil called the "meander". A characteristic consensus sequence known as the P450 "signature" FXXGXXXCXG, located on the C-terminus of the heme binding region, contains a conserved cysteine that serves as a fifth ligand to the heme iron. There are two other conserved motifs specific of the P450 proteins. One is the DGXXT domain, which corresponds to the proton transfer groove on the distal site of the heme. Another is the EXXR domain, which is probably needed to stabilize the core structure located on the proximal side of heme (Werck-Reichhart & Feyereisen, 2000).

### *Microssomal / mitochondrial*

In insects both mitochondrial and microssomal P450 systems have been described. The majority of P450 in insects are microssomal, located in the endoplasmatic reticulum, and require the flavoprotein NADPH cytochrome P450 reductase as the main electron donor; however cytochrome $b_5$ is sometimes needed, depending of the substrate and of the P450 cytochrome involved. Mitochondrial P450 are also present, but, differently from microssomal P450, require ferridoxin and a NADPH ferridoxin reductase as electron donor (Scott & Wen, 2001).

### *Characterization / Function*

Cytochromes P450 enzymes catalyse thousands of different reactions, which are based on the activation of molecular oxygen, with insertion of one of its atoms into the substrate, and reduction of the other to form water (Guengerich, 1991). P450s use electrons from NAD(P)H to catalyse the activation of molecular oxygen, leading to the regiospecific and stereospecific oxidative attack of structurally diverse chemicals (Werck-Reichhart & Feyereisen, 2000).

The interaction that occurs between P450 cytochromes and the NADPH cytochrome P450 reductase is better expressed as a cyclic reaction (Guenguerich, 1991) as it is depicted in Figure 1.

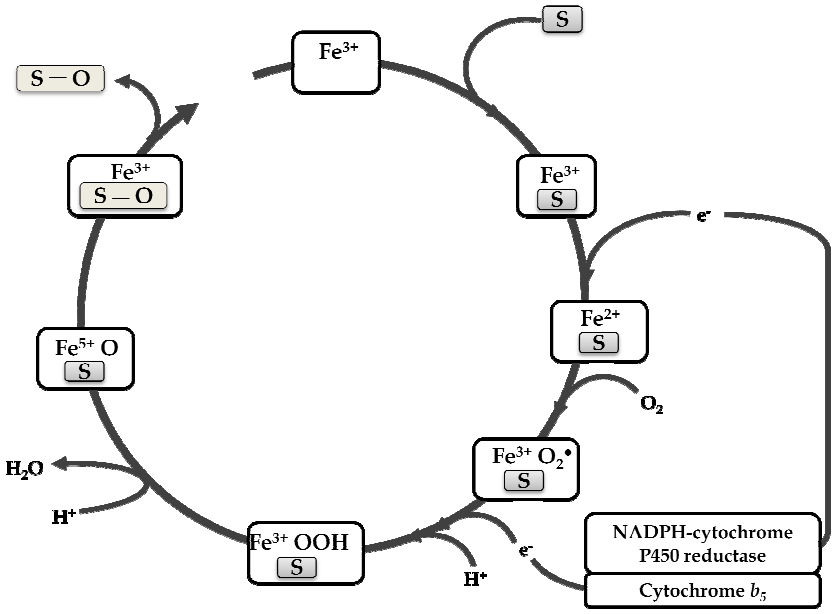



**Figure 1.** Catalytic mechanism of P450 enzymes, where S is the substrate.

The cycle is initiated by the binding of the substrate to the ferric form of the enzyme to form an enzyme-substrate complex, followed by a reduction of the ferric complex by an electron transferred from NADPH via NADPH-cytochrome P450 reductase. Next, the binding of molecular oxygen to the reduced complex forms an enzyme-oxygen-substrate complex followed by the transference of a second electron from NADPH via NADPH-cytochrome P450 reductase or from cytochrome $b_5$. A second proton is added,

which results in the breaking of the oxygen-oxygen bond, releasing one atom of oxygen as water. The oxygen atom remaining is transferred to the substrate, originating an oxidized product, which is released, and a ferric form of the enzyme is once more generated. Then the cycle is re-initiated (Guengerich, 1991).

***Diversity and specificity***

The huge diversity of P450 cytochromes is probably due to an extensive process of gene duplication and cases of gene amplification, conversion, genome duplication, gene loss and lateral transfer (Werck-Reichhart & Feyereisen, 2000). Due to their extremely diverse functions, they can be found with different patterns of expression in all types of tissues and in almost all types of organisms. Although being expressed in a wide range of tissues, insect P450s have their highest activity associated with midgut, fat body and malpighian tubules (Feyereisen, 1999; Scott, 1999).

Additionally, P450s metabolise a large number of substrates, probably due to the existence of numerous P450 isoforms and to the broad specificity of some isoforms (Scott & Wen, 2001). Nevertheless the substrate specificity and type of reaction catalysed by each P450 cytochrome is still not well understood.

Their diversity enables individual P450 cytochromes to display different expression patterns related to life stages, tissues, inducers/inhibitors and substrates. Specifically, there are P450s that are expressed in all life stages (*CYP12* genes) while others are only expressed in adults (*CYP6Z1*) or in larval stages (*CYP6Z3*) (Nikou et al., 2003). Although being found expressed in almost all types of tissues, there are P450s which are tissue specific, while others are everywhere (Feyereisen, 1999; Scott et al., 1998; Scott & Wen, 2001). Expression of P450 cytochromes may also be sex specific, as some P450s showed higher levels of expression in male compared with female tissues (Muller et al., 2007; Nikou et al., 2003).

A large variation in substrate specificity can also be found among different P450s, some being capable of metabolising several substrates while others have only one known substrate (Scott et al., 1998; Scott, 1999). There can be also some overlapping substrate specificity among P450 cytochromes, so that one compound could be metabolised by several enzymes. The production of one or several metabolites from a single substrate

also differs depending on the P450s. P450s show a vast variation in response to inducers and inhibitors, each P450 can be induced/inhibited by one or several compounds. Some P450s can also remain unaltered while others are induced or repressed (Scott et al., 1998).

## *Anopheles gambiae* P450 cytochromes and insecticide resistance

The *A. gambiae* genome has 111 annotated P450 cytochromes (Ranson et al., 2002). The great interest in these cytochromes derives from their role in the oxidative metabolism of insecticides, but only in few cases a definitive link between an increased expression of a specific P450 cytochrome and increased insecticide metabolism has been established.

Increasing reports of specific *A. gambiae* P450 cytochromes being involved in insecticide resistance have been published in the past. The involvement of P450s in pyrethroid resistance started to be demonstrated in *A. gambiae* from Kenyan villages, in synergistic studies using specific P450 cytochrome inhibitors and also given the detection of increased heme levels in resistant mosquitoes (Vulule et al., 1999).

In 2003, Nikou et al., verified that a P450 cytochrome (*CYP6Z1*) was over-expressed in a pyrethroid-resistant strain of *A. gambiae,* and the development of her work pointed to an implication of the involvement of this P450 in conferring pyrethroid resistance to this mosquito (Nikou et al., 2003).

Later, a microarray *chip* was constructed containing fragments from 230 genes associated with detoxification (David et al., 2005) to further study the metabolic based insecticide resistance in *A. gambiae*. From this work resulted the identification of, among other genes, several P450 cytochromes that were highly expressed in the *A. gambiae* permethrin or DDT-resistant strains (David et al., 2005). Of notice is the P450 cytochrome *CYP325A3,* which belongs to a class that was not associated with insecticide resistance before and which was highly over-expressed in an *A. gambiae* permethrin resistant strain. Additionally, *CYP325A3* was later reported as constitutively

over-expressed in a Nigerian pyrethroid resistant strain of *A. gambiae* (Awolola et al., 2009).

In 2007, studies regarding a recently colonised strain of *A. gambiae* from Ghana identified genes whose expression levels were associated with pyrethroid resistance. Among these were three P450 cytochromes (*CYP6M2, CYP6Z2* and *CYP6Z3*) (Muller et al., 2007). These results, together with their location within a cluster of P450 cytochromes in the right arm of chromosome 3 (3R), which is in close association with with a pyrethroid resistance QTL (Ranson et al., 2004), strongly support their involvement in insecticide resistance. A subsequent study showed that *CYP6Z2* displays broad substrate specificity, which may be associated with xenobiotics metabolism and detoxification (Mclaughlin et al., 2008). Despite, *CYP6Z2* being able to bind to permethrin and cypermethrin, *CYP6Z2* does not metabolise neither one of these insecticides (Mclaughlin et al., 2008).

In 2008, Djouaka et al. also identified several P450 cytochromes over-expressed in one or more pyrethroid resistant populations of *A. gambiae.* Among these were *CYP6P3* and once again *CYP6M2*. Both genes showed high levels of over-expression in all the resistant populations, but the first was the gene that showed greatest differences. In the same year, *CYP6P3* was also identified as being up-regulated in another highly permethrin resistant *A. gambiae* population (Müller et al., 2008).

Recent studies on *A. gambiae* recombinant proteins *CYP6M2* (Stevenson et al., 2011) and *CYP6P3* (Müller et al., 2008) demonstrated that these enzymes could metabolise pyrethroids. Thus, the up regulation of these P450 cytochromes in pyrethroid resistant populations, strongly supports a key role for these genes to confer pyrethroid resistance in *A. gambiae*.

Highly expressed P450s have been also reported in DDT resistant strains of *A. gambiae* (David et al., 2005). *CYP6Z1* and *CYP12F1*were strongly over-expressed together with other genes, suggesting that multiple genes could contribute to the DDT resistance phenotype. The slightly over-expression of the electron donor cytochrome P450 reductase in the DDT resistant strain further supported a P450-based resistance mechanism in *A. gambiae* (David et al., 2005).

As the above P450 cytochromes, *CYP314A1* was also found to be over-expressed in a DDT resistant strain of *A. gambiae* from Kenia (Vontas et al., 2005), suggesting a possible involvement in the insecticide resistance phenotype. Both *CYP6Z1* and *CYP6Z2* were over-expressed in DDT resistant strains of *A. gambiae* (David et al. 2005). Although being very similar, these two cytochromes have predicted substrate cavities dramatically different and *CYP6Z1* was predicted to be the only one capable of metabolizing DDT. Chiu et al. (2008) through biochemical characterisations supported these predictions and identified *CYP6Z1* as the only P450 cytochrome capable of metabolising DDT, demonstrating its potential as a target to reduce *A. gambiae* resistance to DDT (Chiu et al., 2008).

Another evidence of the involvement of P450s in insecticide resistance is the fact that silencing the main electron donor of P450 cytochromes, the cytochrome P450 reductase, by RNAi, greatly increased the susceptibility of *A. gambiae* to permethrin, emphasising the important chemoprotective role of P450 cytochromes in this process (Lycett et al., 2006).

Nevertheless, although P450s have been clearly associated with insecticide resistance, the identification of specific P450 cytochromes responsible for insecticide resistance is still extremely difficult.

## *Anopheles gambiae* P450 cytochromes and malaria infection

P450 cytochromes have also been implicated in other vital processes as in *A. gambiae* response to bacterial challenge and to parasite invasion, but the real importance and function of these cytochromes in this process is still not well understood.

A genome expression analysis of *A. gambiae* was made to identify which genes responded to injury, bacterial challenge and malaria infection (Dimopoulos et al., 2002). This analysis identified three P450 cytochromes, one associated with injury, microbial challenge and oxidative stress; the second associated with the response to septic injury which is identical to a bacterial infection *in vivo*; and the third associated with the

response to malaria infection and the presence of lipopolysaccharide (Dimopoulos et al., 2002).

The involvement of P450 cytochromes in response to microbial challenge was established when two P450 cytochromes (*CYP4C27* and *CYP306A1*) were differently expressed in the presence of Gram – (*Salmonella thyphimurium*) or Gram + (*Staphylococcus aureus*) bacteria (Aguilar et al., 2005). This involvement was even more evident when a study, trying to implicate the mosquito midgut microbiota in the defense against malaria parasites, showed that there were ten P450s differently expressed in response to *Escherichia coli* and *S. aureus* in the *A. gambiae* midgut twelve hours after an uninfected blood meal (Dong et al., 2009). Between the P450 cytochromes differently expressed there were *CYP4H17, CYP6M3, CYP6AG1, CYP9J5*, two of them were mitochondrial cytochromes, *CYP49A1* and *CYP12F4* (Dong et al., 2009).

Regarding the relation between P450 cytochromes and the response to malaria infection, it was partly unveiled for the first time in a study about the midgut epithelial responses during *Plasmodium* invasion (Vlachou et al., 2005). Here, several P450 cytochromes were differentially expressed between different phases of the midgut invasion (before invasion, during invasion and after invasion) and also when they compared *Plasmodium* wild-type infection with *Plasmodium* that were unable to invade the epithelium infection (Vlachou et al., 2005). P450s that stood out in this study were *CYP305A1, CYP304B1, CYP6Z1* and *CYP6M4* (Vlachou et al., 2005). The role of P450 cytochromes in the *A. gambiae* response to malaria infection has been reinforced in the last years. Comparing the *A. gambiae* response to two different *Plasmodium* parasites - *P. berghei* and *Plasmodium falciparum* - showed that the mosquito induced slightly different immune responses to each parasite, and that the mosquito was capable of sensing infected blood constituents and mount an immune response, even in the absence of invading ookinetes (Dong et al., 2006). It stands out that, although there were different responses between the three types of infection, in all of them there were P450s differentially expressed in the midgut (*CYP6AG1, CYP6M4, CYP6M1, CYP9J5* and *CYP12F3*) and in the fat body (*CYP6AG1* and *CYP4G17*), suggesting, once more, their involvement in the response to malaria infection.

Further evidence of the link between P450 cytochromes and the mosquito's response to malaria infection came from different studies. First, the effect on gene regulation of the presence of chloroquine in an uninfected blood meal and in a *Plasmodium* infected blood meal was investigated (Abrantes et al., 2008). This work showed that chloroquine affects the abundance of transcripts which encode proteins involved in a variety of processes, including P450 cytochromes that were differently expressed in the *P. berghei* infected blood meal (*CYP9L1, CYP304B1* and *CYP305A1*). A second study focused on the role of *A. gambiae* detoxification enzymes, from the three major families involved in detoxification, GSTs, carboxylesterases and P450 cytochromes, in the response to *Plasmodium* infection (Félix et al., 2010). In this study the impact of *P. berghei* infection was analysed at two time points: one day following the blood meal, during which parasites invade the midgut epithelium, and eleven days after the blood meal when sporozoites were starting to be released to the hemolymph; in two different tissues, midgut and fat body. At day one after the *Plasmodium* infected blood meal they found 17 P450 cytochromes down-regulated and 5 P450 cytochromes up-regulated, including *CYP9L1, CYP304B1, CYP325H1, CYP6M2* and *CYP6Z2* in the midgut, and 5 P450 up-regulated and 1 down-regulated in the fat body, including *CYP12F2, CYP6M2, CYP6M3* and *CYP4G17*. At eleven days after an infected blood meal they found 2 P450 cytochromes up-regulated and 3 down-regulated in the midgut and 1 P450 cytochrome up-regulated and 1 down-regulated in the fat body. The high number of P450 cytochromes differently expressed by the presence of *P. berghei* parasites in different phases of infection and in different tissues suggests that P450 cytochromes are deeply involved in the mosquito response to *Plasmodium* infection, having an important role in different development stages of the parasite and covering different tissues of the mosquito. More specifically, these P450 cytochromes might have a direct role in *Plasmodium* response during the parasite invasion of the midgut epithelium as this is the moment and tissue where more P450 were differentially expressed. The over expression of these P450 cytochromes could be part of a mosquito response mechanism to parasite invasion occurring in the midgut. One possibility is P450s being involved in the cytoskeleton rearrangement (Vlachou et al., 2005; Vlachou & Kafatos, 2005), or P450s could be involved in the production of nitric oxide and other reactive oxygen radicals that are induced by *Plasmodium* invasion of the midgut epithelium (Han et al., 2000;

Luckhart et al., 1998). The blood meal *per si* generates metabolic changes that are also expected to increase the oxidative stress in the mosquito midgut, which is augmented by the presence of *Plasmodium* parasites (Molina-Cruz et al., 2008). Moreover, other parasite killing mechanisms also induce oxidative stress inside the host which, although helping to eliminate the parasite, are also toxic to the host cell. The high level of oxidative stress inside the host cell could trigger cellular and molecular regulation of these P450 cytochromes, at this time point, being responsible for host detoxification together with parasite elimination.

Mosquito hemocytes mediate important cellular immune responses including phagocytosis and encapsulation and also secrete immune factors such as melanization factors and antimicrobial peptides. Recently, studies were made to characterize the role of *A. gambiae* hemocytes in mosquito immunity, consisting in a genome-wide transcriptomic analysis of adult female hemocytes following infection by bacteria and *Plasmodium* parasites (Baton et al., 2009). This work showed that some P450 cytochromes were differently expressed (*CYP325H1* and *CYP6M1*) in the presence of *Micrococcus luteus*, a Gram-positive bacteria (Baton et al., 2009), reinforcing the role of P450 cytochromes in response to microbial challenge described above. This work also showed that a P450 cytochrome was differently expressed 24 hours after the infected blood meal (*CYP325H1*), during *P. berghei* ookinete invasion of the midgut epithelium. Moreover, there were also P450s differentially expressed 19 days after the infected blood meal (*CYP6AG1* and *CYP6M3*), during *P. berghei* sporozoite migration through the hemolymph (Baton et al., 2009), suggesting that P450 cytochromes have a role in the response to malaria infection achieved by hemocytes. Another study aiming to analyse the transcriptional expression and immune functions of circulating hemocytes in naïve and *P. berghei* infected *A. gambiae* females assessed the roles in development of *P. berghei* of many genes expressed in hemocytes (Pinto et al., 2009). Among these genes there were several P450s differently expressed (*CYP6Z1, CYP6M2, CYP6M3* and *CYP12F2*) at 24 - 28 hours after an infected blood meal (Pinto et al., 2009), valuing the importance of this P450 cytochromes in the response to invasion of malaria parasite by hemocytes.

## Conclusion

The role of P450 cytochromes during *Plasmodium* invasion is still poorly understood, but it may play out to be of utmost importance to combat malaria transmission. Here, we intend to bring an update review on the connection between P450 cytochromes and the *A. gambiae* response to malaria infection, identifying several P450 cytochromes that probably are, directly or indirectly, involved in the response to *Plasmodium* invasion. We have also reviewed the implication of P450 cytochromes in *A. gambiae* insecticide resistance. However, uncovering the objective role of these cytochromes in insecticide resistance, that is naming specific cytochromes and describing in detail the processes in which those specific P450s are involved is still extremely difficult.

The consistent detection of differential expression of P450 cytochromes, in studies about either insecticide resistance or the response to malaria infection, suggests that the role of these P450s could be similar in these two processes. Nevertheless, the real importance and function of P450 cytochromes in these processes is still not well understood neither the possibility of interplay between infection and insecticide resistance. One of the P450 cytochromes with expression altered in response to insecticides and *Plasmodium* infection was *CYP6M2* that, was also highly over-expressed in a pyrethroid-resistant strain of *A. gambiae* mosquitoes (Muller et al., 2007) and highly over-expressed in response to *Plasmodium* infection in both the midgut and the fat body 1 day after an infected blood meal (Félix et al., 2010). These results suggest that the role of *CYP6M2* might be the same in response to insecticides and infection, or that these two processes might share the activation mechanism of *CYP6M2* expression. *CYP6M2* could also function as an endogenous mediator, acting as the first response to different challenges, which would explain being increased by parasite infection and insecticide exposure. Similar to *CYP6M2* is *CYP6Z1*, yet another P450 cytochrome that is over-expressed in insecticides-resistant strains of *A. gambiae* (Nikou et al., 2003, David et al., 2005) and is also over-expressed in response to *Plasmodium* infection (Vlachou et al., 2005). The increase in the expression of this P450 could function as an immediate response to an exogenous challenge or *A. gambiae* could have the same mechanism of response, including over-expression of specific P450 cytochromes, to parasite infection and insecticide exposure. *CYP6Z2* was highly over-expressed in a pyrethroid-resistant strain (Müller et al., 2007), but opposite to *CYP6M2*, was down-

regulated in the midgut of *A. gambiae* at day 1 and day 11 after an infected blood meal (Félix et al., 2010). These results suggest a different role for *CYP6Z2* in response to the insecticide and to parasite infection, however we have to take into account that, although being able to bind to permethrin and cypermethrin, *CYP6Z2* does not metabolise these compounds (Mclaughlin et al., 2008). So the over-expression of *CYP6Z2* in a pyrethroid-resistant strain might be associated with different processes other than insecticide resistance.

A more complete knowledge about the factors involved in P450 cytochromes response to malaria infection and insecticide resistance is extremely needed for the implementation of efficient malaria and vector control programmes, including strategies able to adapt to different types of resistance. Although the interaction of insecticides with P450 enzymes has been studied, many of its aspects still remains poorly understood. Grasping the underlying processes in this interaction might help mitigate the problem of insecticide resistance, and therefore contribute to the control of malaria and other human diseases.

## References

Abrantes, P.; Dimopoulos, G.; Grosso, A.R.; do Rosário, V.E. & Silveira, H. (2008). Chloroquine mediated modulation of *Anopheles gambiae* gene expression. *PLoS One*, 3(7), e2587, ISSN 1932-6203

Aguilar, R.; Dong, Y.; Warr, E. & Dimopoulos, G. (2005). *Anopheles* infection responses; laboratory models versus field malaria transmission systems. *Acta Tropica*, 95(3), 285-291, ISSN 0001-706X

Awolola,T.S.; Oduola, O.A.; Strode, C.; Koekemoer, L.L.; Brooke, B. & Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae* sensu stricto from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 103, 1139-1145, ISSN 0035-9203

Baton, L.A.; Robertson, A.; Warr, E.; Strand, M.R. & Dimopoulos, G. (2009). Genome-wide transcriptomic profiling of *Anopheles gambiae* hemocytes reveals pathogen-specific signatures upon bacterial challenge and *Plasmodium berghei* infection. *BMC Genomics*, 5, 10:257, ISSN 1471-2164

Brogdon, W.G. & McAllister, J.C. (1998). Insecticide resistance and vector control. *Emerging Infectious Diseases,* 4(4), 605-613, ISSN: 1080-6059

Chiu, T.L.; Wen, Z.; Rupasinghe, S.G. & Schuler, M.A. (2008). Comparative molecular modeling of *Anopheles gambiae* CYP6Z1, a mosquito P450 capable of metabolizing DDT. *Proceedings of the National Academy of Sciences*, 105(26), 8855-8860, ISSN 0027-8424

David, J.P.; Strode, C.; Vontas, J.; Nikou, D.; Vaughan, A.; Pignatelli, P.M.; Louis, C.; Hemingway, J. & Ranson, H. (2005). The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences*, 102(11), 4080-4084, ISSN 0027-8424

Dimopoulos, G.; Christophides, G.K.; Meister, S.; Schultz, J.; White, K.P.; Barillas-Mury, C. & Kafatos, F.C. (2002). Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection.

*Proceedings of the National Academy of Sciences*, 99(13), 8814-8819, ISSN 0027-8424

Djouaka, R.F.; Bakare, A.A.; Coulibaly, O.N.; Akogbeto, M.C.; Ranson, H.; Hemingway, J. & Strode, C. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae s.s*. from Southern Benin and Nigeria. *BMC genomics*, 9, 538, ISSN 1471-2164

Dong, Y.; Aguilar, R.; Xi, Z.; Warr, E.; Mongin, E. & Dimopoulos, G. (2006). *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathogens*, 2(6), e52, ISSN 1553-7366

Dong, Y.; Manfredini, F. & Dimopoulos, G. (2009). Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathogens*, 5(5), e1000423, ISSN 1553-7366

Dunkov, B.C.; Guzov, V.M.; Mocelin, G.; Shotkoski, F.; Brun, A.; Amichot, M.; Ffrench-Constant, R.H. & Feyereisen, R. (1997). The *Drosophila* Cytochrome P450 Gene Cyp6a2: Structure, Localization, Heterologous Expression, and Induction by Phenobarbital. DNA and Cell Biology, 16(11), 1345-1356, ISSN 1044-5498

Enayati, A.A.; Ranson, H. & Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology*, 14(1), 3-8, ISSN 0962-1075

Félix, R.C.; Müller, P.; Ribeiro, V.; Ranson, H. & Silveira, H. (2010). *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. *BMC genomics*, 11, 312, ISSN 1471-2164

Feyereisen, R. (1999). Insect P450 Enzymes. *Annual Review of Entomology*, 44, 507-533, ISSN 0066-4170

Ffrench-constant, R.H.; Pittendrigh, B.; Vaughan, A. & Anthony, N. (1998). Why are there so few resistance-associated mutations in insecticide target genes? *Phil.Trans. R. Soc. Lond. B*, 353, 1685-1693, ISSN 1471-2970

Guengerich, F.P. (1991). Reactions and Significance of Cytochrome P-450 Enzymes. *The Journal of Biological Chemistry*, 266(16), 10019-10022, ISSN 0021–9258

Han, Y.S.; Thompson, J.; Kafatos, F.C. & Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *Embo Journal*, 19(22), 6030-6040, ISSN 0261-4189

Hemingway, J. & Karunaratne, S.H. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and veterinary Entomology*, 12(1), 1-12, ISSN 1365-2915

Hemingway, J. & Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology*. 41, 371-391, ISSN 0066.4170

Hemingway, J.; Hawkes, N.J.; McCarrol, L. & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, 34, 653-655, ISSN 0965-1748

Kumar, S.; Christophides, G.K.; Cantera, R.; Charles, B.; Han, Y.S.; Meister, S.; Dimopoulos, G.; Kafatos, F.C. & Barillas-Mury, C. (2003). The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 100(24), 14139-14144, ISSN 0027-8424

Kumar, S.; Gupta, L.; Han, Y.S. & Barillas-Mury, C. (2004). Inducible peroxidases mediate nitration of *anopheles* midgut cells undergoing apoptosis in response to *Plasmodium* invasion. *Journal of Biological Chemistry*, 279(51), 53475-53482, ISSN 0021-9258

Kasai, S. & Scott, J.G. (2000). Overexpression of Cytochrome P450 CYP6D1 Is Associated with Monooxygenase-Mediated Pyrethroid Resistance in House Flies from Georgia. *Pesticide Biochemistry and Physiology*, 68, 34-41, ISSN 0048-3575

Luckhart, S.; Vodovotz, Y.; Cui, L. & Rosenberg, R. (1998). The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric

oxide. *Proceedings of the National Academy of Sciences,* 95(10), 5700-5705, ISSN 0027-8424

Lycett, G.J.; McLaughlin, L.A.; Ranson, H.; Hemingway, J.; Kafatos, F.C.; Loukeris, T.G. & Paine, M.J. (2006). Anopheles gambiae P450 reductase is highly expressed in oenocytes and in vivo knockdown increases permethrin susceptibility. *Insect Molecular Biology*, 15(3), 321-327, ISSN 0962-1075

Martinez-Torres, D.; Chandre, F.; Williamson, M.S.; Darriet, F.; Bergé, J.B.; Devonshire, A.L.; Guillet, P.; Pasteur, N. & Pauron, D. (1998). Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s.*. *Insect Molecular Biology*, 7(2), 179-184, ISSN 0962-1075

McLaughlin,L.A.; Niazi, U.; Bibby, J.; David, J.P.; Vontas, J.; Hemingway, J.; Ranson, H.; Sutcliffe, M.J. & Paine, M.J. (2008). Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Molecular Biology*, 17(2), 125-135, ISSN 0962-1075

Molina-Cruz, A.; DeJong, R.J.; Charles, B.; Gupta, L.; Kumar, S., Jaramillo-Gutierrez, G. & Barillas-Mury, C. (2008) Reactive Oxygen Species Modulate *Anopheles gambiae* Immunity against Bacteria and *Plasmodium*. *The Journal of Biological Chemistry*, 283(6), 3217-3223, ISSN 0021–9258

Müller, P.; Donnelly, M.J. & Ranson, H. (2007). Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. *BMC genomics*, 8, 36, ISSN 1471-2164

Müller,P.; Warr, E.; Stevenson, B.J.; Pignatelli, P.M.; Morgan, J.C.; Steven, A.; Yawson, A.E.; Mitchell, S.N.; Ranson, H.; Hemingway, J.; Paine. M.J. & Donnelly, M.J. (2008). Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genetics*, 4(11), e1000286, ISSN 1553-7390

Mutero, A.; Pralavorio, M.; Bride, J.M. & Fournier, D. (1994). Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proceedings of the National Academy of Sciences*, 91(13), 5922-5526, ISSN 0027-8424

Nebert, D.W.; Nelson, D.R.; Coon, M.J.; Estabrook, R.W.; Feyereisen, R.; Fujii-Kuriyama, Y.; Gonzalez, F.J.; Guengerich, F.P.; Gunsalus, I.C.; Johnson, E.F.; Loper, J.C.; Sato, R.; Waterman, M.R. & Waxman, D.J. (1991). The P450 Superfamily: Update on New Sequences, Gene Mapping, and Recommended Nomenclature. *DNA and Cell Biology,* 10(1), 1-14, ISSN 1044-5498

Nelson, D.R.; Koymans, L.; Kamataki, T.; Stegeman, J.J; Feyereisen, R.; Waxman, D.J.; Waterman, M.R.; Gotoh, O.; Coon, M.J.; Estabrook, R.W.; Gunsalus, I.C. & Nebert, D.W. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, 6(1), 1-42, ISSN 0960-314X

Newcomb, R.D.; Campbell, P.M.; Ollis, D.L.; Cheah, E.; Russell, R.J. & Oakeshott, J.G. (1997). A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proceedings of the National Academy of Sciences*, 94(14), 7464-7468, ISSN 0027-8424

N'Guessan, R.; Darriet, F.; Guillet, P.; Carnevale, P.; Traore-Lamizana, M.; Corbel, V.; Koffi, A.A. & Chandre, F. (2003). Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase. *Medical and veterinary Entomology*, 17(1), 19-25, ISSN 1365-2915

Nikou, D.; Ranson, H. & Hemingway, J. (2003). An adult-specific CYP6 P450 gene is overexpressed in a oyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene*, 318, 91-102, ISSN 0378-1119

Ortelli, F.; Rossiter, L.C.; Vontas, J.; Ranson, H. & Hemingway, J. (2003). Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. *Biochemical Journal*, 373, 957-963, ISSN 0264-6021

Peterson, T.M.; Gow, A.J. & Luckhart, S. (2007). Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. *Free Radical Biology & Medicine*, 42(1), 132-142, ISSN 0891-5849

Pinto, S.B.; Lombardo, F.; Koutsos, A.C.; Waterhouse, R.M.; McKay, K.; Chunju, A.; Ramakrishnan, C.; Kafatos, F.C. & Michel, K. (2009). Discovery of *Plasmodium* modulators by genome-wide analysis of circulating hemocytes in *Anopheles gambiae*. *Proceedings of the National Academy of Sciences,* 106(50), 21270-21275, ISSN 0027-8424

Ranson, H.; Jensen, B.; Vulule, J.M.; Wang, X.; Hemingway, J. & Collins, F.H. (2000a). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Molecular Biology, 9 (5), 491-497, ISSN 0962-1075

Ranson, H.; Jensen, B.; Wang, X.; Prapanthadara, L.; Hemingway, J. & Collins, F.H. (2000b). Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Biology*, 9(5), 499-507, ISSN 0962-1075

Ranson, H.; Rossiter, L.; Ortelli, F.; Jensen, B.; Wang, X.; Roth, C.W.; Collins, F.H. & Hemingway, J. (2001). Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochemical Journal*, 359, 295-304, ISSN 0264-6021

Ranson, H.; Claudianos, C.; Ortelli, F.; Abgrall, C.; Hemingway, J.; Sharakhova, M.V.; Unger, M.F.; Collins, F.H. & Feyereisen, R. (2002). Evolution of supergene families associated with insecticide resistance. *Science*, 298, 179-181, ISSN 0036-8075

Ranson, H.; Paton, M.G.; Jensen, B.; McCarroll, L.; Vaughan, A.; Hogan, J.R.; Hemingway, J. & Collins, F.H. (2004). Genetic mapping of genes conferring permethrin resistance in the malaria vector, *Anopheles gambiae*. *Insect Molecular Biology*, 13 (4), 379-386, ISSN 0962-1075

Sabourault, C.; Guzov, V.M.; Koener, J.F.; Claudianos, C.; Plapp. F.W.Jr. & Feyereisen, R. (2001). Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. *Insect Molecular Biology*, 10 (6), 609-618, ISSN 0962-1075

Scott, J.G.; Liu, N. & Wen, Z. (1998). Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comparative Biochemistry and Phisiology Part C*, 121(1-3), 147-155, ISSN 1532-0456

Scott, J.G. (1999). Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology*, 29(9), 757-777, ISSN 0965-1748

Scott, J.G. & Wen, Z. (2001) Cytochromes P450 of insects: the tip of the iceberg. *Pest Management Science*, 57(10), 958-967, ISSN 1526-4998

Stevenson, B.J.; Bibby, J.; Pignatelli, P.; Muangnoicharoen, S.; O'Neill, P.M.; Lian, L.Y.; Müller, P.; Nikou, D.; Steven, A.; Hemingway, J.; Sutcliffe, M.J. & Paine, M.J. (2011). Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology*, in press, ISSN 0965-1748

Vlachou, D. & Kafatos, F.C. (2005). The complex interplay between mosquito positive and negative regulators of *Plasmodium* development. *Current Opinion in Microbiology*, 8(4), 415-421, ISSN 1369-5274

Vlachou, D.; Schlegelmilch, T.; Christophides, G.K. & Kafatos, F.C. (2005). Functioal Genomic Analysis of Midgut Epithelial Responses in *Anopheles* during *Plasmodium* Invasion. *Current Biology*, 15(13), 1185-1195, ISSN 0960-9822

Vontas, J., Blass, C. Koutsos, A.C., David, J.-P., Kafatos, F.C., Louis, C., Hemingway, J., Christophides, G.K. & Ranson, H. (2005). Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology*, 14(5), 509-521, ISSN 0962-1075

Vulule, J.M., Beach, R.F., Atieli, F.K., McAllister, J.C., Brogdon, W.G., Roberts, J.M., Mwangi, R.W. & Hawley, W.A. (1999). Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenyan villages using permethrin-impregnated nets. *Medical and veterinary Entomology*, 13(3), 239-244, ISSN 1365-2915

Werck-Reichhart, D. & Feyereisen, R. (2000). Cytochromes P450: a success history. *Genome Biology*, 1(6), 3003, ISSN 1759-6653

Wilce, M.C. & Parker, M.W. (1994). Structure and function of glutathione S-transferases. *Biochim Biophys Acta*, 1205, 1-18, ISSN 0006-3002

World Health Organization (2010). World Malaria Report 2010, ISBN 978 92 4 156410. Geneve.

Yassine H. & Osta M.A. (2010). *Anopheles gambiae* innate immunity. *Cellular Microbiology*, 12(1), 1-9, ISSN 1462-5814

Zhu, Y.C.; Dowdy, A.K. & Baker, J.E. (1999). Differential mRNA expression levels and gene sequences of a putative carboxylesterase-like enzyme from two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Insect Molecular Biology*, 29(5), 417-421, ISSN 0962-1075

# Chapter 3 - *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression

**This chapter was published as a research paper:**

Felix R.C., Muller P., Ribeiro V., Ranson H., Silveira H.. (2010) *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. *BMC Genomics* 11: 312

Félix et al. BMC Genomics 2010, 11:312
http://www.biomedcentral.com/1471-2164/11/312

BMC Genomics

RESEARCH ARTICLE Open Access

# *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression

Rute C Félix[1], Pie Müller[2,3,4], Vera Ribeiro[5], Hilary Ranson[2], Henrique Silveira[1*]

## Abstract

**Background:** *Anopheles gambiae* has been shown to change its global gene expression patterns upon *Plasmodium* infection. While many alterations are directly related to the mosquito's innate immune response, parasite invasion is also expected to generate toxic by-products such as free radicals. The current study aimed at identifying which loci coding for detoxification enzymes are differentially expressed as a function of *Plasmodium berghei* infection in midgut and fat body tissues.

**Results:** Using a custom-made DNA microarray, transcript levels of 254 loci primarily belonging to three major detoxification enzyme families (glutathione S-transferases, cytochrome P450 monooxygenases and esterases) were compared in infected and uninfected mosquitoes both during ookinete invasion and the release of sporozoites into the hemocoel. The greatest changes in gene expression were observed in the midgut in response to ookinete invasion. Interestingly, many detoxification genes including a large number of P450s were down-regulated at this stage. In the fat body, while less dramatic, gene expression alterations were also observed and occurred during the ookinete invasion and during the release of sporozoites into the hemocoel. While most gene expression changes were tissue-related, *CYP6M2*, a CYP previously associated with insecticide resistance, was over-expressed both in the midgut and fat body during ookinete invasion.

**Conclusions:** Most toxicity-related reactions occur in the midgut shortly after the ingestion of an infected blood meal. Strong up-regulation of *CYP6M2* in the midgut and the fat body as well as its previous association with insecticide resistance shows its broad role in metabolic detoxification.

## Background

The mosquito *Anopheles gambiae* is the main malaria vector in sub-Saharan Africa. Resistance to anti-malaria drugs and insecticides together with the lack of vaccines highlight the need for novel strategies in malaria control. Such a strategy could be the interruption of the transmission cycle within the mosquito.

The mosquito becomes infected with the malaria parasite by taking a blood meal. The blood meal itself brings metabolic changes and induces a state of oxidative stress [1,2]. This is further increased by the presence of *Plasmodium* parasites in the blood meal [3]. During mosquito response to infection, active nitrogen and oxygen radicals are produced to contain *Plasmodium* infection [1,3]. These products may represent potential oxidative stress that can be ameliorated or eliminated by detoxification enzymes. For example several glutathione S-transferases (GSTs) have peroxidase activity and some can also metabolise reactive α,β-aldehydes [4]. GST expression can also be induced by reactive oxygen species (ROS) [5,6]. While GSTs help to eliminate ROS, cytochrome P450 monooxygenases (CYP) may actually contribute towards its generation [7].

Although transcription alteration of detoxification genes in response to bacteria and *Plasmodium* [8-10] has been described, the nature of this response hasn't been fully discussed. In this study we describe the impact of *P. berghei* infection at two time points (1 day and 11 days post infection) on the expression of detoxification genes in the midgut and fat body. We identified several genes, previously implicated in the detoxification of xenobiotics, which are differentially expressed in relation to parasite infection in the midgut and fat body.

* Correspondence: hsilveira@ihmt.unl.pt
[1]Centro de Malária e Outras Doenças Tropicais, UEI Malária, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, 96, 1349-008 Lisbon, Portugal

The possible role of detoxification enzymes in modulating malaria transmission is discussed.

## Results and Discussion

### Microarray

Tissues for microarray analyses were collected at two critical time points of the *Plasmodium* cycle in the mosquito host: 1 day following the blood meal, during which parasites invade the midgut epithelium, and 11 days after the blood meal when sporozoites are starting to be released to the hemolymph, as demonstrated by detection of parasite's DNA in the hemolymph (data not shown). The mosquitoes were fed on mice that were either infected with the parasite or uninfected. The success of infection was indirectly confirmed by randomly selecting up to 19-44 mosquitoes that were screened for the presence of oocysts (see Table S1 in Additional file 1). Most of the mosquitoes were found to be positive (70.5% to 84%) and hence it can be assumed that the tissues used in the gene expression studies were infected too.

The microarray experiment was developed to answer the following questions, regarding midgut and fat body tissues:

1. which genes respond to *Plasmodium* midgut epithelium invasion (1 day post blood meal)
2. which genes respond to the release of sporozoites into the hemolymph (11 days post blood meal), and
3. which genes respond differently between the two events (interaction term).

In the microarray analysis 146 loci were differentially expressed in at least one of the comparisons made. The results for all comparisons are given in Table S2 (Additional file 2). The microarray results were validated by comparing the mean values for the expression data ($\log_2$ ratio) for genes from three independent replicates obtained by microarray analysis with the corresponding mean expression values obtained with the multiplex quantitative RT-PCR. The Pearson correlation coefficient ($P$ = 0.884 for midgut, $P$ = 0.85 for fat body) demonstrates a high degree of correlation between the two methods (see Figure S1 in Additional file 3).

### Genes differentially expressed in infected *versus* uninfected mosquitoes at day 1 post blood meal

At day 1 post blood meal more changes were observed in the midgut as compared to the fat body. While in the midgut 54 genes were differentially expressed, only 13 were different in the fat body (Figure 1, Table 1). In the midgut, 22 CYPs were differentially expressed with the majority (17) being down-regulated. In the fat body, five out of the six CYPs differentially expressed in response to *Plasmodium* infection were up-regulated. The vast majority of these differentially expressed CYPs belong to families primarily associated with detoxification roles (e.g. *CYP4*, *CYP6* and *CYP9*) rather than families implicated in hormone biosynthetic pathways [11]. Similarly in the GST family the two classes primarily associated with xenobiotic detoxification, Delta and Epsilon [4], were generally repressed in response to parasite infection with the notable exception of *GSTD5* which was strongly up-regulated (> 8.5 ×) in infected *vs.* uninfected midguts.

In both *A. gambiae* and *A. stephensi*, *Plasmodium* parasite invasion induces an increase of nitric oxide synthase (NOS) expression and in turn an increase in nitric oxide (NO) and NO metabolites [12-15]. NO has been shown to down regulate CYP gene expression in other organisms [16]. We hypothesize therefore that the observed down-regulation of CYPs in the midgut may also be linked to increased levels of NO.

Of the up-regulated CYPs, *CYP6M2* showed the greatest response to infection (Figure 2). This gene has already been reported to be over-expressed in response to *P. berghei* infection [10] and implicated in resistance to pyrethroid insecticides [17,18]. One possible explanation for this up-regulation is a response to an endogenous mediator increased upon the infection process. As an example, prostaglandins have been shown to induce expression of CYPs in human liver cells [19].

Superoxide dismutases constitute part of the first line of defence against ROS and reactive nitrogen oxide species (RNOS) [1]. However, *SOD2* was down-regulated 1 day post infection suggesting that down-regulation of oxidative stress response genes could be part of the defence response triggered by parasite invasion. A similar mechanism has been described for other oxidative stress response genes such as catalase in response to *Plasmodium* invasion [3].

Cytoskeleton reorganization and up-regulation of genes related to folding and movement of microtubules suggest that cytoskeleton dynamics and remodelling function as key elements of *Plasmodium* invasion of the *Anopheles* midgut [2]. This epithelium rearrangement is a robust molecular response to ookinetes penetration. In a whole genome microarray study seven tubulins were differentially up-regulated during the invasion period [2]. Here too, three cytoskeletal genes represented on the Detox array, *tubulin B*, *tubulin A* and *actin*, were up-regulated at day 1 post infection (1.85, 8.76 and 1.44 fold, respectively). In mammals, microtubule disruption leads to down-regulation of several CYPs [20] and perhaps similar responses also lead to down-regulation of CYPs during parasite invasion.

Félix et al. BMC Genomics 2010, 11:312
http://www.biomedcentral.com/1471-2164/11/312



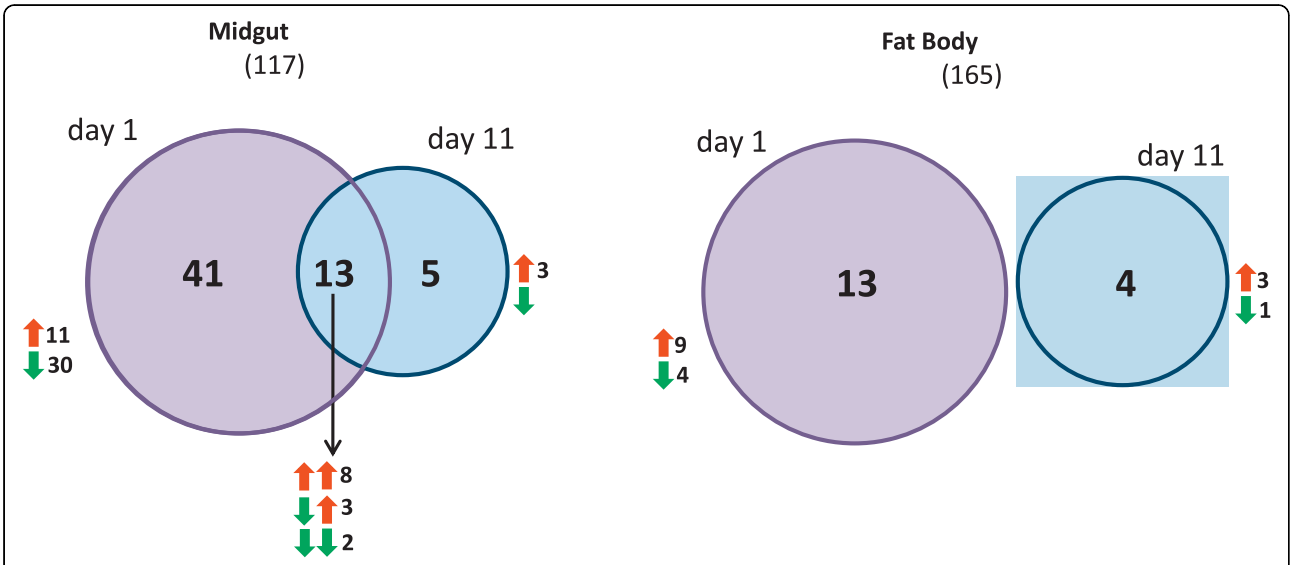



**Figure 1 Differential expression of detoxification genes in the midgut and fat body at day 1 and day 11 post feeding with a *P. berghei* infected or an uninfected blood meal**. The most dramatic change occurs in the midgut during sporozoite invasion (day 1 post blood feed) with 33 loci being down-regulated. While genes were predominantly down-regulated in the midgut the majority of differentially expressed genes in the fat body are up-regulated during midgut infection and sporozoite release. Numbers in brackets indicate the number of loci that were not differentially expressed at the significance cut-off level of *alpha* = 0.001.

### Genes differentially expressed in infected *versus* uninfected mosquitoes 11 days post blood meal

At this time point, when sporozoites are released from oocysts to the hemocoel a less pronounced effect on the transcript levels of detoxification genes was observed as compared to midgut invasion (Table 1). Two of the genes up-regulated on day 11, *CYP4H25* and *CYP4H15*, were down-regulated at day 1 (Table 1 and 2), suggesting that their suppression is linked to the invasion of the midgut epithelium by the parasite, while their up-regulation on day 11 may be associated with subtle changes in midgut structure as sporozoites are leaving oocysts. *GST01* was up-regulated at both time points (Table 1 and 2) which indicates that this enzyme is directly involved in the response to parasites at both stages. Although at this stage the fat body would have had direct contact with parasites or at least molecules released by parasites during midgut egress, the transcriptional response in the fat body was more pronounced at day 1 than on day 11 post infection. *CYP6M2* was down-regulated on day 11 but was up-regulated at day 1 (Table 1 and 2), indicating that this CYP responds to particular events of the parasites life cycle.

### Genes that show a different response between *Plasmodium* midgut epithelium invasion and release of sporozoites into the hemolymph

The interaction term between the two time points was investigated to compare responses to *Plasmodium* invasion of the midgut epithelium (day 1) and to the release of sporozoites into the hemolymph (11 days). Heat diagrams with the genes that presented significant positive (increased relative expression from day 1 to day 11) and negative interaction (decreased relative expression from day 1 to day 11) in midgut and fat body are shown in Figure 2. The number of genes under positive interaction was higher in the midgut while the opposite was seen in the fat body, reflecting the active site of infection.

ABC transporters from family c showed a strong negative interaction in the midgut and to a lesser extent in the fat body, implying that these cytoplasmic membrane transporters are important for infection control probably by transporting glutathione conjugates or lipid-derived eicosanoids that are known to be involved in insect response to infection [21].

The interaction analysis confirmed that there is a considerable difference between the gene expression levels between day 1 and day 11 in response to *Plasmodium* infection. There were a high number of genes that had different levels of expression in response to the ookinetes invasion of the midgut and in response to the release of sporozoites in the hemolymph, showing that these genes have the ability of changing their expression levels according with the time of infection.

In the midgut, the majority (69%) of differentially expressed genes between day 11 and day 1 were the same both in uninfected and infected mosquitoes, as was the direction of change, indicating that these

Félix *et al. BMC Genomics* 2010, **11**:312
http://www.biomedcentral.com/1471-2164/11/312



**Table 1 Genes differentially expressed ($p < 0.001$) between infected and uninfected mosquitoes on day 1 after infection**

| Gene description | Probe name | 1 day | | | |
|---|---|---|---|---|---|
| | | Midgut fold | *P-value* | Fat Body fold Fold | *P-value* |
| **ABC transporter** | ABCC10 | 2.38 | 0 | | |
| | ABCC11 | 3.94 | 0 | | |
| | ABCC12 | 1.77 | 0 | | |
| **Actin** | Actin5C | 1.44 | 0.0008 | | |
| **Cytochrome P450 monooxygenase** | CYP12F2 | -1.93 | 0 | 2.19 | 0 |
| | CYP12F4 | -2.12 | 0 | | |
| | CYP304B1 | -1.96 | 0 | | |
| | CYP325H1 | -1.86 | 0.0003 | | |
| | CYP4AR1 | -1.96 | 0.0004 | | |
| | CYP4D15 | -2.70 | 0 | | |
| | CYP4G17 | | | -1.26 | 0.00095 |
| | CYP4H15 | -1.79 | 0 | | |
| | CYP4H17 | -2.79 | 0 | | |
| | CYP4H25 | -2.06 | 0 | | |
| | CYP6AA1 | -1.82 | 0 | | |
| | CYP6AA2 | -1.93 | 0 | | |
| | CYP6AH1 | -2.44 | 0 | | |
| | CYP6M1 | 1.60 | 0 | | |
| | CYP6M2 | 4.23 | 0 | 2.73 | 0 |
| | CYP6M3 | 1.62 | 0 | 2.10 | 0 |
| | CYP6M4 | -1.29 | 0 | | |
| | CYP6P1 | -1.38 | 0.0004 | | |
| | CYP6Y1 | | | 1.61 | 0 |
| | CYP6Y2 | 1.73 | 0 | | |
| | CYP6Z2 | -2.80 | 0 | | |
| | CYP9J3 | -1.83 | 0 | | |
| | CYP9L1 | -1.46 | 0 | | |
| | CYP9M1 | 1.52 | 0.0004 | 1.43 | 0 |
| **Esterase** | COEAE6G | -1.52 | 0.00099 | | |
| | COEunkn | | | 2.19 | 0.0003 |
| **Glutathione peroxidase** | GPX2B | | | 1.55 | 0 |
| **Glutaredoxin** | GRX1 | 1.53 | 0 | | |
| **Glutathione S-transferase** | GSTD1_5 | -1.56 | 0 | | |
| | GSTD2 | -1.67 | 0 | | |
| | GSTD3 | -1.55 | 0 | 2.17 | 0 |
| | GSTD5 | 8.62 | 0.0006 | | |
| | GSTD6 | -1.65 | 0 | | |
| | GSTD11 | 1.48 | 0 | | |
| | GSTD12 | -1.49 | 0 | | |
| | GSTE2 | -1.57 | 0.0001 | | |
| | GSTE3 | -1.51 | 0 | | |
| | GSTE7 | -1.84 | 0 | | |
| | GSTE8 | 1.57 | 0 | | |
| | GSTO1 | 2.90 | 0 | | |
| | GSTMS1 | -1.46 | 0 | | |
| | GSTMS3 | -1.36 | 0 | | |
| | GSTS1_2 | 2.08 | 0 | | |
| | GSTT2 | -1.25 | 0.0002 | | |
| | GSTU2 | 1.91 | 0 | | |

**Table 1: Genes differentially expressed ($p < 0.001$) between infected and uninfected mosquitoes on day 1 after infection** *(Continued)*

| | | | | | |
|---|---|---|---|---|---|
| | GSTZ1 | | | -1.42 | 0 |
| **Midgut maltase-like protein** | AGM1 | -1.59 | 0 | -1.59 | 0 |
| **NADPH P450 reductase** | NADPH_P450_red | | | -1.53 | 0.0002 |
| **Nitrilase** | NIT8537 | | | 2.54 | 0 |
| **Ribosomal protein** | RPL19 | -1.37 | 0 | | |
| | RPS26 | -1.53 | 0 | | |
| **Salivary gland protein** | GSG8 | -1.43 | 0.0002 | | |
| **Superoxide dismutase** | SOD2 | -1.98 | 0 | | |
| **Thioredoxin peroxidase** | TPX3 | -1.47 | 0 | | |
| | TPX4 | 1.26 | 0.0004 | | |
| **Tubulin** | TubulinA | 1.85 | 0 | | |
| | TubulinB | 8.76 | 0 | | |

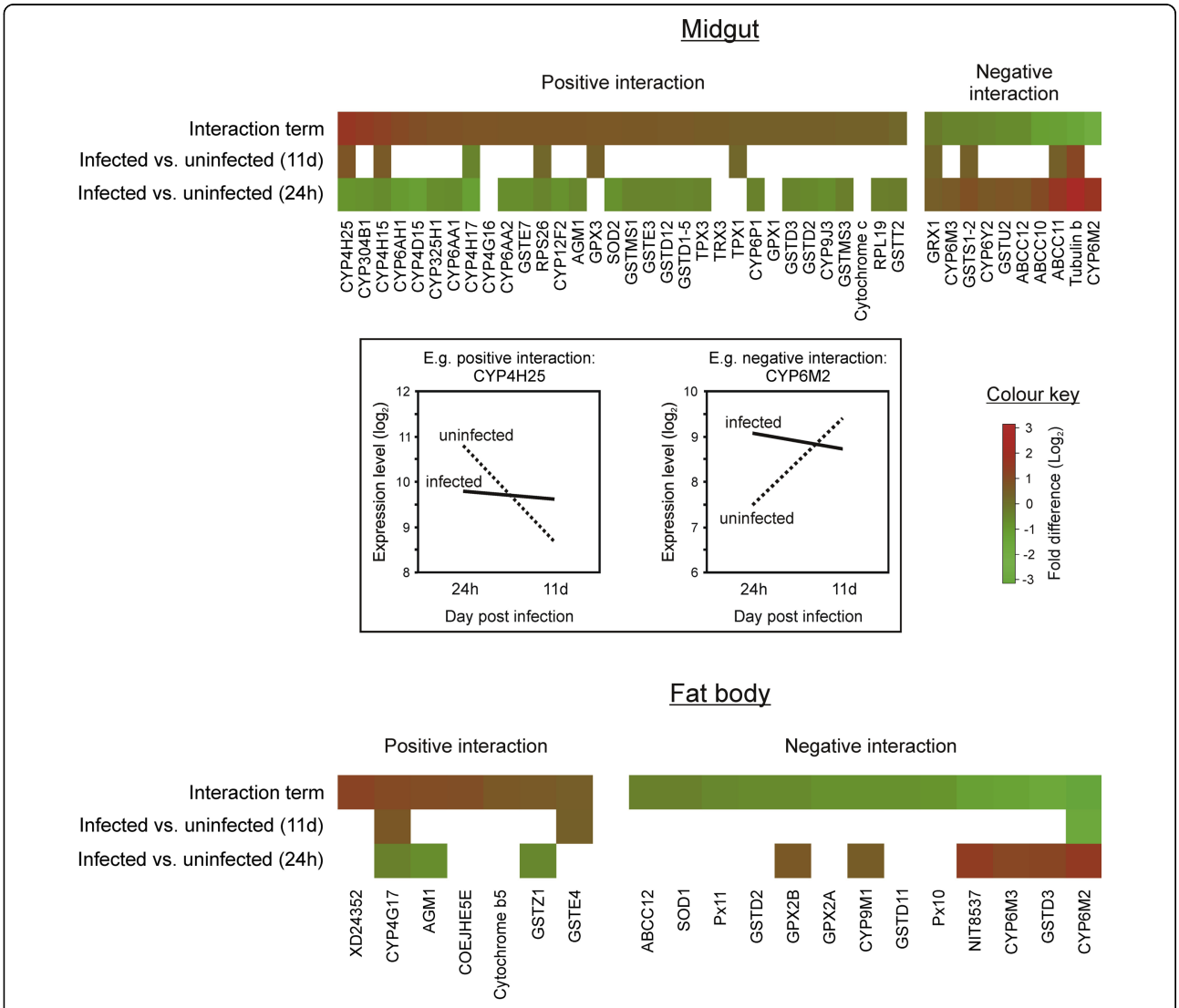



**Figure 2 Heat diagrams showing genes that responded differently between the event of *Plasmodium* invasion into the midgut epithelium (day 1 post feeding) and the release of sporozoites into the hemolymph (day 11 post feeding)**. The loci are plotted in the top rows and arranged from most positive interaction on the left (red) to most negative interaction (green). Inset: Examples for a positive and a negative interaction term observed in the midgut. Only loci that showed a significant interaction term ($p < 0.001$) are plotted.

Félix et al. BMC Genomics 2010, 11:312
http://www.biomedcentral.com/1471-2164/11/312



**Table 2 Genes differentially expressed ($p < 0.001$) between infected and uninfected mosquitoes on day 11 after infection**

| Gene description | Probe name | 11 days | | | |
|---|---|---|---|---|---|
| | | Midgut fold | *P-value* | Fat Body fold | *P-value* |
| **ABC transporter** | ABCC11 | 1.47 | 0.0009 | | |
| **Cytochrome P450 monooxygenase** | CYP4G17 | | | 1.58 | 0 |
| | CYP4H15 | 1.65 | 0 | | |
| | CYP4H17 | -1.44 | 0.0002 | | |
| | CYP4H19 | -1.65 | 0.0001 | | |
| | CYP4H25 | 1.89 | 0.0004 | | |
| | CYP6M2 | | | -2.91 | 0 |
| | CYP6Z2 | -1.97 | 0 | | |
| **Glutathione peroxidase** | GPX3 | 1.49 | 0 | | |
| **Glutaredoxin** | GRX1 | 1.28 | 0 | | |
| **Glutathione S-transferase** | GSTD10 | -1.65 | 0.0008 | | |
| | GSTD11 | 1.70 | 0 | | |
| | GSTE4 | | | 1.35 | 0.0003 |
| | GSTO1 | 2.22 | 0 | | |
| | GSTS1_2 | 1.43 | 0 | | |
| **Ribosomal protein** | RPS26 | 1.23 | 0 | | |
| **Thioredoxin peroxidase** | TPX1 | 1.27 | 0.0004 | | |
| | TPX2 | 1.34 | 0 | | |
| | TPX4 | 1.55 | 0 | 1.65 | 0.0001 |
| **Tubulin** | TubulinA | 1.55 | 0 | | |
| | TubulinB | 2.61 | 0 | | |

genes were responding mainly to the blood meal, as it represents a strong oxidative insult. However, this total concordance was not observed in the fat body where only 26% of genes were regulated in the same direction between infected and uninfected while 38% were regulated in opposite directions (see Table S2 in Additional file 2). The trend of expression of both tissues suggests that differences observed are due to fat body response to parasite released from the oocysts into the hemocoel.

The mosquito response to sporozoites in the hemolymph triggers effector mechanisms like melanization [1], and a burst of expression of genes encoding constituents of the immune system including the production of free radicals [12] that needs a counter detoxification reaction. After excluding genes similarly regulated in both infected and uninfected groups, fat body CYP genes were down-regulated, at day 11, as observed for the midgut at day 1. *SOD2* was down-regulated and seems to be determinant for parasite control. *TPX4* was up-regulated confirming its role on infection detoxification mediated by the fat body. The fat body has an important role in the detoxification and in the immune response of the mosquito on day 11 of infection when compared with day 1 post infection, which is not observed when we compare infected and uninfected mosquitoes on day 11.

## Conclusions

This study determined transcription profiles of detoxification enzymes during *Plasmodium* infection in *A. gambiae*, showing important changes in the expression of several detoxification enzymes, as well as membrane associated ABC transporters. Interestingly, genes coding for detoxification enzymes revealed a variable response, being differentially induced or repressed depending on the tissue and stage of infection.

Although the mechanism underlying these changes is presently unclear, this differential regulation of detoxification genes observed during *Plasmodium* infection may be due to 1) the increasing oxidative stress caused by the presence of the parasite; 2) the epithelium rearrangement involving alterations in cytoskeleton genes caused by the ookinetes invasion and the oocysts burst; or 3) a combination of both. A hypothetical scenario for the inter-relationship between infection and detoxifying molecules is depicted in Figure 3.

In contrast to the majority of differentially expressed P450s which were down-regulated in response to midgut invasion, *CYP6M2* expression was induced in

Félix et al. BMC Genomics 2010, 11:312
http://www.biomedcentral.com/1471-2164/11/312



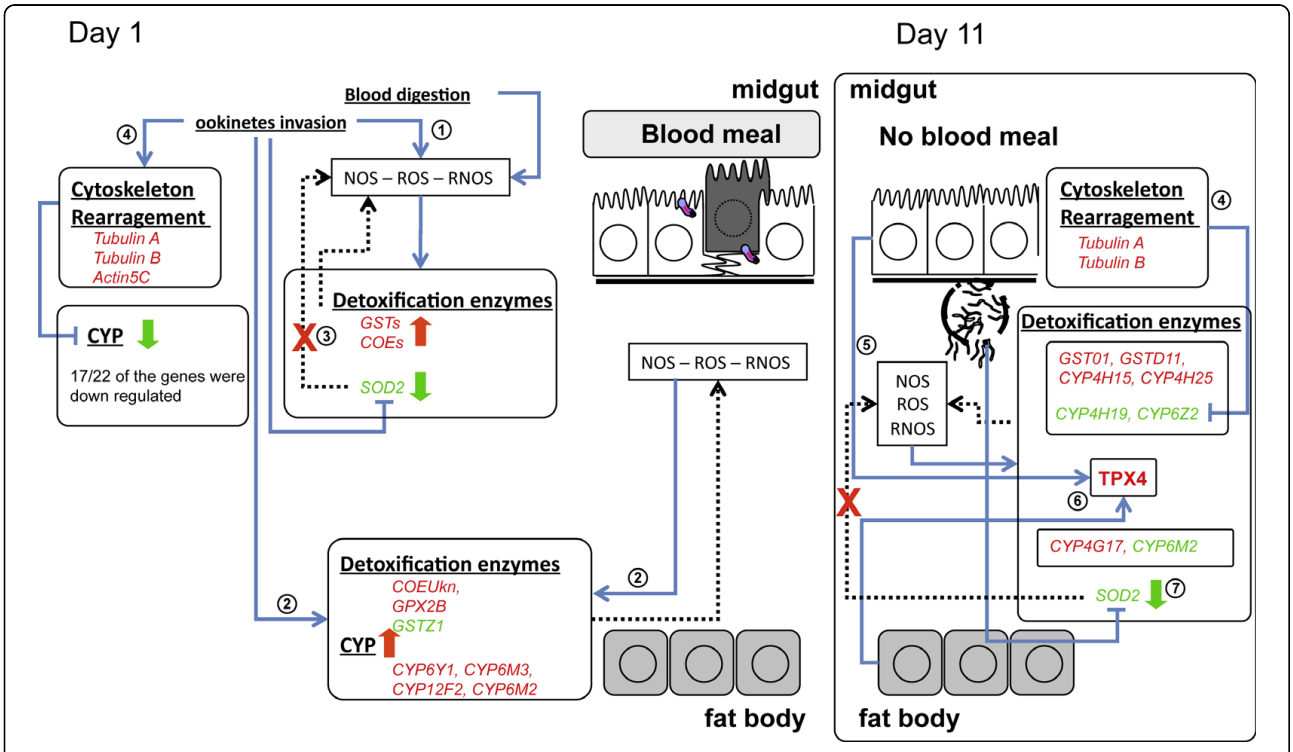



**Figure 3 Hypothetical scenario of *Anopheles gambiae* detoxification response to *Plasmodium berghei* infection**. Midgut and fat body genes up-regulated (red) and down-regulated (green) after infection at 2 different time points, day 1 (invasion of midgut epithelium by ookinetes) and day 11 (sporozoite egress from oocysts), and between the two events. At day 1, blood digestion and parasite invasion cause an increase in the ROS and RNOS that consequently increases the expression of detoxification enzymes (1). The parasite invasion and the ROS and RNOS also affect the fat body increasing the expression of detoxification enzymes in this tissue (2). At the same time midgut cells in response to parasite invasion suppresses the SOD expression (3) as a mechanism to eliminate parasites. Parasite invasion of midgut epithelium causes a massive cytoskeleton rearrangement that down regulates CYPs expression (4). On day 11, there is no blood digestion, but oocysts burst and sporozoites are released to the hemolymph. In the midgut the oocysts burst provokes a cytoskeleton rearrangement (4), as in day 1, that probably also down regulates CYPs expression in the midgut. While in the hemolymph sporozoites cause an increase in the ROS and RNOS that increase the detoxification enzymes expression in both midgut and fat body (5). Here, both midgut and fat body altered the expression of *TPX4* (6), that is essential for hydrogen peroxide detoxification through the thioredoxin system. In the fat body sporozoites also provoke suppression of SOD expression (7).

response to *P. berghei* infection. This CYP is involved in resistance to pyrethroid insecticides [17,18]. This observation together with regulation of other genes, such as membrane ABC transporters involved in xenobiotic elimination, lead us to speculate that there might be an association between the response to *Plasmodium* infection and insecticide resistance, enhancing the importance of further studying their interaction.

## Methods

### Mosquitoes

*A. gambiae* s.s. (molecular M form) of the Yaoundé strain mosquitoes were reared at 26°C and 75% relative humidity on a 12/12 hours light/dark cycle. Adult mosquitoes were maintained on 10% glucose solution until blood feeding.

### *P. berghei* infection of mosquitoes

Female CD1 mice were intraperitoneally inoculated with $10^7$ *P. berghei* ANKA parasitised red blood cells. The levels of parasitaemia were measured from blood samples of the mouse tail using Giemsa-stained blood films. When the parasitaemia reached 10-20% and exflagellation was observed, mice were used to infect mosquitoes. Female mosquitoes were allowed to feed directly on naïve (control) and *P. berghei* infected mice up to one hour, with regular monitoring to certify mice were anesthetised. Fully engorged mosquitoes were kept at 19-21°C and 80% relative humidity for P. berghei development. The maintenance and care of experimental animals complied with portaria n° 1005/92 from 23rd October and was approved by the Divisão Geral de Veterinaria, Portugal.

### Tissue collection

Mosquito midguts and abdominal walls containing fat body tissues were collected from pools of 40 sibling mosquitoes at day 1 and on day 11 after the blood meal. This procedure was repeated to obtain 3 independent replicates. Tissues were dissected from mosquitoes submerged in ice-cold phosphate-buffered saline (PBS) that was prepared with DEPC-treated water and transferred to ice-cold RNAlater (Ambion). After incubation at 4°C over night any excess RNAlater was removed and samples were stored at -20°C until RNA extraction. On day 11 post infection mosquito midguts were also collected to determine infection rate (number of infected mosquitoes over total number of mosquitoes observed).

### Microarray analysis

Protocols for RNA extraction, amplification and labelling with fluorescent dyes are described in [22]. Fluorescent Cy3- and Cy5-labelled targets were hybridised to the latest version of the *A. gambiae detox chip* [23] (ArrayExpress accession AMEXP-863). The features on this version of the *detox chip* probe for 103 cytochrome P450s, 31 esterases, 35 glutathione S-transferases and 85 additional loci coding for enzymes such as peroxidases, reductases, superoxide dismutases, ATP-binding cassette transporters, tissue specific genes and housekeeping genes.

Two separate microarray experiments were conducted; one for the RNA pools obtained from midguts and another one for RNA extracted from fat body tissues. Each experiment followed a 2 × 2 factorial design in which the first factor was *time* and the second one was *infection status*. Each factor was measured at two levels; at one and eleven days post blood meal and from female mosquitoes that were either fed with *Plasmodium*-infected or uninfected blood. Factors and levels were combined constituting a total of four measurements. Each combination was repeated three times with tissues from 40 individuals pooled for RNA isolation per replicate (see Figure S2 in Additional file 4).

After scanning of raw signal intensities and visual spot inspection in GenePix Pro 5.1 software (Axon Instruments) data were exported to *limma* (version 2.9). *Limma*, part of the Bioconductor project [24], is a bioinformatics package for the analysis of linear models in microarray experiments [25] implemented in R http://www.r-project.org. Here, median spot and background intensities from the red (Cy5) and green (Cy3) channels were analysed. Any spot with a saturated signal in either the green or the red channel was excluded from the statistical analysis. For each spot, background intensities were first subtracted from the foreground intensities. To generate positive corrected intensities any intensity that was less than 0.5 after background subtraction was reset to 0.5. Background-corrected intensities from each spot were then transformed to intensity log-fold changes, $M = \log_2(red) - \log_2(green)$, and their geometrical means, $A = [\log_2(red) + \log_2(green)]/2$. Within each array, $M$-values for each spot were subsequently normalized as a function of $A$ using the loess scatter plot smoothing function implemented in *limma*. In the normalization step the calibration spots on the *detox chip* were included too. The *detox chip* contains 40 calibration spots representing a 1:1 dilution series over a concentration gradient from 1 pg to 30 ng per 2 μl of added mRNA spike-in mix).

For the statistical analysis of the microarray experiments *limma* employs a linear model approach whereby linear models are fitted to the normalised data for each locus probed by the array [25,26]. Because each unique probe is spotted four times onto the *detox chip* we took advantage of the pooled correlation method implemented in *limma* to make full use of the replicate spots [27]. Contrasts, linear combinations of the coefficients, were then tested for significance. The contrasts tested between factor levels (*time* and *infection status*) and the interaction term (*time* × *infection status*) are given in Figure S2 (Additional file 4). To assess differential expression *limma* uses an empirical Bayes method to moderate the standard errors of the estimated log-fold changes [26]. This approach results in more stable inference and improved power, especially for experiments with small numbers of arrays [28]. $P$-values obtained from the $t$-tests (with the moderated $t$-statistic) were adjusted for multiple testing adopting the approach of Benjamini and Hochberg [25,29]. In order to define a set of differentially expressed genes only hits with an adjusted $p$-value below the level of significance, $\alpha = 0.001$, were considered.

All microarray data have been deposited in ArrayExpress (ArrayExpress accession E-MTAB-195).

### Quantitative RT-PCR

To validate microarray data a subset of 20 differentially expressed genes (see Table S2 in Addditional file 2) were chosen and their expression levels measured by multiplexed quantitative RT-PCR. The same RNA pools used in the microarray experiment served as target RNA in the PCR. The Beckman Coulter GeXP system was used to quantify the expression of these genes and the ribosomal protein RPS7-encoding gene [VectorBase: AGAP010592] was used for normalisation as described in [22]. PCR primer sequences are given in Table S3 (see Additional file 5).

**Additional file 1: Table S1**. Infection rate and oocyst load of *A. gambiae* infected with *P. berghei* used for the microarray experiments.

**Additional file 2: Table S2**. List of all the genes differentially expressed ($p < 0.001$) represented on the *Detox* chip including fold change in expression and *p*-values.

**Additional file 3: Figure S1**. Validation of the DNA microarray analysis using quantitative RT-PCR. The mean expression values for midgut genes (A) and fat body genes (B) obtained by microarray analysis were plotted against the corresponding mean expression values obtained with quantitative RT-PCR. A high level of consistency between the two datasets was demonstrated by the Pearson correlation coefficient ($P = 0.884$) for midgut and ($P = 0.85$) for fat body and best-fit linear-regression analysis ($R^2 = 0.7814$) for midgut and ($R^2 = 0.7228$) for fat body.

**Additional file 4: Figure S2**. Design of the microarray experiments. The experiments for midgut and fat body tissues followed the same layout. The boxes of the graphs represent RNA extracted from pools of 40 individuals and the arrows the microarrays to which labelled target RNA was co-hybridized. The tails of the arrows represent the samples that were labelled with a green (Cy3) and the heads those samples that were labelled with a red (Cy5) fluorescent dye. For the design matrix in *limma*, the samples from uninfected tissues collected 1 day post infection were set as the reference pool (shaded boxes). After fitting linear models the contrasts shown below the diagram were constructed for hypothesis testing of specific comparisons between RNA pools. For each of the three biological blocks (replicates 1 to 3) and factor combination a separate coefficient was included in the design matrix. The contrasts were extracted by taking the average of the three comparisons.

**Additional file 5: Table S3**. Sequences of oligonucleotide primers used in quantitative RT-PCR validation experiments.

**Acknowledgements**
This work was partially supported by Fundação para a Ciência e a Tecnologia (FCT), trough research funds from project POCTI/IMI/59489/2004. RF is a recipient of a FCT grant (SFRH/BD/28024/2006).

**Author details**
[1]Centro de Malária e Outras Doenças Tropicais, UEI Malária, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, 96, 1349-008 Lisbon, Portugal. [2]Vector Group, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK. [3]Vector Control Unit, Medical Department, Swiss Tropical and Public Health Institute, CH-4002 Basel, Switzerland. [4]University of Basel, CH-4003 Basel, Switzerland. [5]Centro de Biomedicina Molecular e Estrutural (CBME), Instituto de Biotecnologia e Bioengenharia (IBB-LA), Universidade do Algarve, Faro, Portugal.

**Authors' contributions**
RF and PM performed the experiments; RF, PM and HS analyzed and interpreted the data; RF, PM, HR and HS wrote the paper. RF, PM, HS and VR conceived and designed the experiments. All authors read and approved the final manuscript.

Received: 6 November 2009 Accepted: 19 May 2010
Published: 19 May 2010

**References**

1. Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C: **The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae.** *Proc Natl Acad Sci USA* 2003, **100**:14139-14144.
2. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC: **Functional genomic analysis of midgut epithelial responses in Anopheles during Plasmodium invasion.** *Curr Biol* 2005, **15**:1185-1195.
3. Molina-Cruz A, DeJong RJ, Charles B, Gupta L, Kumar S, Jaramillo-Gutierrez G, Barillas-Mury C: **Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium.** *J Biol Chem* 2008, **283**:3217-3223.
4. Ranson H, Hemingway J: **Mosquito glutathione transferases.** *Methods Enzymol* 2005, **401**:226-241.
5. Ortelli F, Rossiter LC, Vontas J, Ranson H, Hemingway J: **Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector Anopheles gambiae.** *Biochem J* 2003, **373**:957-963.
6. Vontas JG, Small GJ, Hemingway J: **Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens.** *Biochem J* 2001, **357**:65-72.
7. Morgan ET: **Regulation of cytochromes P450 during inflammation and infection.** *Drug Metab Rev* 1997, **29**:1129-1188.
8. Aguilar R, Das S, Dong Y, Dimopoulos G: **Continuous exposure to Plasmodium results in decreased susceptibility and transcriptomic divergence of the Anopheles gambiae immune system.** *BMC Genomics* 2007, **8**:451.
9. Baton LA, Robertson A, Warr E, Strand MR, Dimopoulos G: **Genome-wide transcriptomic profiling of Anopheles gambiae hemocytes reveals pathogen-specific signatures upon bacterial challenge and Plasmodium berghei infection.** *BMC Genomics* 2009, **10**:257.
10. Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G: **Anopheles gambiae immune responses to human and rodent Plasmodium parasite species.** *PLoS Pathog* 2006, **2**:e52.
11. Feyereisen R: **Insect P450 enzymes.** *Annu Rev Entomol* 1999, **44**:507-533.
12. Dimopoulos G, Seeley D, Wolf A, Kafatos FC: **Malaria infection of the mosquito Anopheles gambiae activates immune-responsive genes during critical transition stages of the parasite life cycle.** *EMBO J* 1998, **17**:6115-6123.
13. Han YS, Thompson J, Kafatos FC, Barillas-Mury C: **Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes.** *EMBO J* 2000, **19**:6030-6040.
14. Luckhart S, Vodovotz Y, Cui L, Rosenberg R: **The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide.** *Proc Natl Acad Sci USA* 1998, **95**:5700-5705.
15. Peterson TM, Gow AJ, Luckhart S: **Nitric oxide metabolites induced in Anopheles stephensi control malaria parasite infection.** *Free Radic Biol Med* 2007, **42**:132-142.
16. Hara H, Adachi T: **Contribution of hepatocyte nuclear factor-4 to down-regulation of CYP2D6 gene expression by nitric oxide.** *Mol Pharmacol* 2002, **61**:194-200.
17. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, Strode C: **Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria.** *BMC Genomics* 2008, **9**:538.
18. Muller P, Donnelly MJ, Ranson H: **Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana.** *BMC Genomics* 2007, **8**:36.
19. Antoun J, Goulitquer S, Amet Y, Dreano Y, Salaun JP, Corcos L, Plée-Gautier E: **CYP4F3B is induced by PGA1 in human liver cells: a regulation of the 20-HETE synthesis.** *J Lipid Res* 2008, **49**:2135-2141.
20. Dvorak Z, Ulrichova J, Modriansky M: **Role of microtubules network in CYP genes expression.** *Curr Drug Metab* 2005, **6**:545-552.
21. Stanley D: **Prostaglandins and other eicosanoids in insects: biological significance.** *Annu Rev Entomol* 2006, **51**:25-44.
22. Muller P, Chouaibou M, Pignatelli P, Etang J, Walker ED, Donnelly MJ, Simard F, Ranson H: **Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in Anopheles arabiensis sampled from an area of cotton fields in Northern Cameroon.** *Mol Ecol* 2008, **17**:1145-1155.
23. David JP, Strode C, Vontas J, Nikou D, Vaughan A, Pignatelli PM, Louis C, Hemingway J, Ranson H: **The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors.** *Proc Natl Acad Sci USA* 2005, **102**:4080-4084.
24. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G,

Tierney L, Yang JY, Zhang J: **Bioconductor: open software development for computational biology and bioinformatics.** *Genome Biol* 2004, **5**:R80.
25. Smith GK: **Limma: linear models for microarray data.** *Bioinformatics and Computational Biology Solutions using R and Bioconductor* New York: SpringerGentleman R, Carey V, Dudoit S, Irizarry R, Huber W 2005, 397-420.
26. Smyth GK: **Linear models and empirical bayes methods for assessing differential expression in microarray experiments.** *Stat Appl Genet Mol Biol* 2004, **3, Article3.**
27. Smyth GK, Michaud J, Scott HS: **Use of within-array replicate spots for assessing differential expression in microarray experiments.** *Bioinformatics* 2005, **21**:2067-2075.
28. Smyth GK, Yang YH, Speed T: **Statistical issues in cDNA microarray data analysis.** *Methods Mol Biol* 2003, **224**:111-136.
29. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I: **Controlling the false discovery rate in behavior genetics research.** *Behav Brain Res* 2001, **125**:279-284.

doi:10.1186/1471-2164-11-312
**Cite this article as:** Félix *et al.*: *Plasmodium* infection alters *Anopheles gambiae* detoxification gene expression. *BMC Genomics* 2010 **11**:312.

## Additional file 1

**Table S1.** Infection rate and oocyst load of *A. gambiae* infected with *P.berghei* used for the microarray experiments.

| | Experiment 1 | Experiment2 | Experiment 3 |
|---|---|---|---|
| N | 44 | 24 | 19 |
| Infection Rate | 70.5 | 75 | 84 |
| Median number of oocysts by midgut | 60 | 70.33 | 50.75 |

# Additional file 2

**Table S2**. List of all the genes differentially expressed (p<0.001) represented on the *Detox* chip including fold change in expression and *p*-values.

| | | | Day 1 vs Day 1 | | | | Day 11 vs Day 11 | | | | Day 11 vs Day 1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | infected vs uninfected | | | | infected vs uninfected | | | | Midgut | | | |
| | | | Midgut | | Fat Body | | Midgut | | Fat Body | | infected | | uninfected | |
| Gene description | Probe name | VectorBase Gene ID | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value |
| ABC transporter | ABCC10 | AGAP008437 | 2,3801 | 0,0000 | | | | | | | | | 1,8302 | 0,0003 |
| ABC transporter | ABCC11 | AGAP008436 | 3,9422 | 0,0000 | | | 1,4722 | 0,0010 | | | 1,9534 | 0,0000 | 5,2307 | 0,0000 |
| ABC transporter | ABCC12 | AGAP007917 | 1,7654 | 0,0000 | | | | | | | 1,7183 | 0,0000 | 2,8919 | 0,0000 |
| ABC transporter | ABCC9 | AGAP008437 | | | | | | | | | 1,9279 | 0,0005 | 2,2863 | 0,0000 |
| Actin | Actin5C | AGAP000651 | 1,4380 | 0,0008 | | | | | | | 1,5084 | 0,0001 | 1,8895 | 0,0000 |
| Aldehyd oxydase | Aldehyd oxydase | AGAP006226 | | | | | | | | | 1,2579 | 0,0003 | | |
| Alpha-amylase | Alpha_amylase | AGAP002317 | | | | | | | | | | | | |
| Cuticle protein | AGAP008432 | AGAP008432 | | | | | | | | | 2,6759 | 0,0007 | 2,5883 | 0,0009 |
| Cytochrome b5 | Cytochrome_B5 | AGAP007121 | | | | | | | | | | | | |
| Cytochrome c | Cytochrome_C | AGAP009537 | | | | | | | | | -1,5316 | 0,0000 | -2,1435 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP12F2 | AGAP008020 | -1,9319 | 0,0000 | 2,19162 | 0 | | | | | 2,2517 | 0,0000 | | |
| Cytochrome P450 monooxygenase | CYP12F4 | AGAP008018 | -2,1170 | 0,0000 | | | | | | | 3,5235 | 0,0000 | 2,2815 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP301A1 | AGAP006082 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP302A1 | AGAP005992 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP303A1 | AGAP010077 | | | | | | | | | 10,0144 | 0,0000 | 9,9452 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP304B1 | AGAP003066 | -1,9616 | 0,0000 | | | | | | | 4,3954 | 0,0000 | | |
| Cytochrome P450 monooxygenase | CYP305A1 | AGAP005656 | | | | | | | | | 2,3054 | 0,0000 | 2,4267 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP306A1 | AGAP004665 | | | | | | | | | -2,0167 | 0,0007 | -2,0237 | 0,0007 |
| Cytochrome P450 monooxygenase | CYP307B1 | AGAP008682 | | | | | | | | | 1,8327 | 0,0000 | 1,8895 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP314A1 | AGAP002429 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP315A1 | AGAP00284 | | | | | | | | | 2,7151 | 0,0000 | 3,0801 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP325A1 | AGAP002211 | | | | | | | | | -2,0720 | 0,0002 | | |
| Cytochrome P450 monooxygenase | CYP325C2 | AGAP002205 | | | | | | | | | | | 2,0849 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP325H1 | AGAP002138 | -1,8635 | 0,0003 | | | | | | | | | -2,7492 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP325J1 | AGAP001443 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP329A1 | AGAP003522 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP4AA1 | AGAP003608 | | | | | | | | | 2,8899 | 0,0000 | 2,0965 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4AR1 | AGAP002417 | -1,9561 | 0,0004 | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP4C27 | AGAP009246 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP4D15 | GenBank: AY062193 | -2,6963 | 0,0000 | | | | | | | 1,4123 | 0,0000 | -1,5562 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4D22 | AGAP002419 | | | | | | | | | 4,1411 | 0,0000 | 4,7305 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4G16 | AGAP001076 | | | | | | | | | -2,1856 | 0,0000 | -4,1728 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4G17 | AGAP000877 | | | -1,2614 | 0,00095 | | | 1,5822746 | 0 | | | | |
| Cytochrome P450 monooxygenase | CYP4H15 | AGAP001864 | -1,7888 | 0,0000 | | | 1,6472 | 0,0000 | | | -4,9314 | 0,0000 | -14,5203 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4H17 | AGAP008358 | -2,7876 | 0,0000 | | | -1,4419 | 0,0002 | | | | | -2,1600 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4H19 | AGAP000088 | | | | | -1,6449 | 0,0001 | | | | | | |

| | | | Day 1 vs Day 1 | | | | Day 11 vs Day 11 | | | | Day 11 vs Day 1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | infected vs uninfected | | | | infected vs uninfected | | | | Midgut | | | |
| | | VectorBase | Midgut | | Fat Body | | Midgut | | Fat Body | | infected | | unifected | |
| Gene description | Probe name | Gene ID | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value | Fold | P-value |
| Cytochrome P450 monooxygenase | CYP4H25 | AGAP001864 | -2,0605 | 0,0000 | | | 1,8934 | 0,0004 | | | -2,4811 | 0,0000 | -9,6800 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4H26 | GenBank: AY748849 | | | | | | | | | 6,7693 | 0,0000 | 6,8828 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4J5 | AGAP006048 | | | | | | | | | | | 3,1910 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP4J9 | AGAP006047 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP4K2 | AGAP002416 | | | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP6AA1 | AGAP002862 | -1,8150 | 0,0000 | | | | | | | 2,7837 | 0,0000 | | |
| Cytochrome P450 monooxygenase | CYP6AA2 | GenBank: AY745221 | -1,9319 | 0,0000 | | | | | | | 2,5901 | 0,0000 | | |
| Cytochrome P450 monooxygenase | CYP6AF1/2 | AGAP011029 | | | | | | | | | -1,6958 | 0,0006 | | |
| Cytochrome P450 monooxygenase | CYP6AG1 | GenBank: AY745223 | | | | | | | | | 2,2784 | 0,0000 | 1,9972 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6AG2 | GenBank: AY745224 | | | | | | | | | 1,6335 | 0,0000 | 1,3140 | 0,0001 |
| Cytochrome P450 monooxygenase | CYP6AH1 | AGAP007480 | -2,4368 | 0,0000 | | | | | | | 2,7511 | 0,0000 | | |
| Cytochrome P450 monooxygenase | CYP6AK1 | AGAP010961 | | | | | | | | | 9,1960 | 0,0000 | 15,7469 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6M1 | AGAP008209 | 1,5966 | 0,0000 | | | | | | | 4,1670 | 0,0000 | 5,5251 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6M2 | AGAP008212 | 4,2310 | 0,0000 | 2,72641 | 0 | | | -2,9059301 | 0 | | | 6,7272 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6M3 | AGAP008213 | 1,6200 | 0,0000 | 2,10235 | 0 | | | | | 3,0823 | 0,0000 | 4,3772 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6M4 | AGAP008214 | -1,2932 | 0,0000 | | | | | | | | | | |
| Cytochrome P450 monooxygenase | CYP6N1 | GenBank: AY028786 | | | | | | | | | 4,2634 | 0,0000 | 10,7108 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6N2 | AGAP008206 | | | | | | | | | 2,5544 | 0,0000 | 2,9404 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6P1 | AGAP002868 | -1,3794 | 0,0004 | | | | | | | 7,9502 | 0,0000 | 5,3889 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6P3 | AGAP002865 | | | | | | | | | 3,2243 | 0,0000 | 3,4082 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6P5 | AGAP002866 | | | | | | | | | 2,4284 | 0,0001 | 2,6611 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6S1 | AGAP008204 | | | | | | | | | 2,2784 | 0,0000 | 2,0648 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6Y1 | AGAP008208 | | | 1,61217 | 0,00002 | | | | | | | 2,1629 | 0,0002 |
| Cytochrome P450 monooxygenase | CYP6Y2 | AGAP008207 | 1,7255 | 0,0000 | | | | | | | 2,6427 | 0,0000 | 4,0954 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6Z1 | AGAP008219 | | | | | | | | | 22,8639 | 0,0000 | 24,3019 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6Z2 | AGAP008218 | -2,8031 | 0,0000 | | | -1,9738 | 0,0000 | | | 8,2363 | 0,0000 | 5,7998 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP6Z4 | AGAP002894 | | | | | | | | | | | 2,4674 | 0,0001 |
| Cytochrome P450 monooxygenase | CYP9J3 | AGAP012291 | -1,8264 | 0,0000 | | | | | | | 3,5702 | 0,0000 | 2,4708 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9J4 | AGAP012292 | | | | | | | | | 27,5504 | 0,0000 | 15,0741 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9J5 | AGAP012296 | | | | | | | | | 31,1681 | 0,0000 | 15,1579 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9K1 | AGAP000818 | | | | | | | | | 3,2355 | 0,0000 | 2,2486 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9L1 | AGAP012295 | -1,4621 | 0,0000 | | | | | | | 1,8609 | 0,0000 | 1,4570 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9L2 | AGAP012294 | | | | | | | | | 1,9172 | 0,0001 | 1,7053 | 0,0001 |
| Cytochrome P450 monooxygenase | CYP9L3 | AGAP012293 | | | | | | | | | | | 2,2115 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9M1 | AGAP009374 | 1,5231 | 0,0004 | 1,42603 | 0,00001 | | | | | 5,5751 | 0,0000 | 6,7599 | 0,0000 |
| Cytochrome P450 monooxygenase | CYP9M2 | AGAP009375 | | | | | | | | | 4,5631 | 0,0007 | 3,9258 | 0,0001 |
| Esterase | COE13O | AGAP011507 | | | | | | | | | | | | |
| Esterase | COE15O | AGAP011576 | | | | | | | | | | | | |
| Esterase | COEAE1F | AGAP006227 | | | | | | | | | 2,3883 | 0,0000 | 1,9793 | 0,0000 |
| Esterase | COEAE2D | AGAP005757 | | | | | | | | | 2,3473 | 0,0000 | 2,6870 | 0,0000 |
| Esterase | COEAE3D | AGAP005758 | | | | | | | | | | | | |

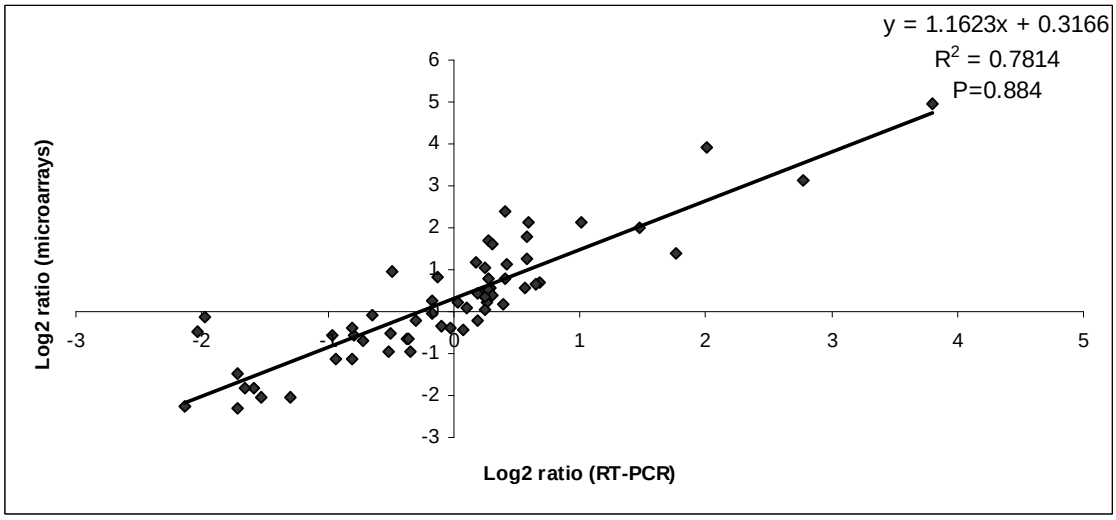
| | | | Day 1 vs Day 1 | | | | Day 11 vs Day 11 | | | | Day 11 vs Day 1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | infected vs uninfected | | | | infected vs uninfected | | | | Midgut | | | |
| | | VectorBase | Midgut | | Fat Body | | Midgut | | Fat Body | | infected | | unifected | |
| **Gene description** | **Probe name** | **Gene ID** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** |
| Esterase | COEAE2F | AGAP006228 | | | | | | | | | 1,5390 | 0,0000 | | |
| Esterase | COEAE2G | AGAP006723 | | | | | | | | | | | | |
| Esterase | COEAE4G | AGAP006725 | | | | | | | | | | | | |
| Esterase | COEAE6G | AGAP006727 | -1,5199 | 0,0010 | | | | | | | | | | |
| Esterase | COEBE2O | AGAP001101 | | | | | | | | | | | | |
| Esterase | COEBE3C | AGAP005372 | | | | | | | | | -1,8038 | 0,0007 | -2,4066 | 0,0000 |
| Esterase | COEJHE2E | AGAP005834 | | | | | | | | | | | | |
| Esterase | COEJHE5E | AGAP005837 | | | | | | | | | | | | |
| Esterase | COEunkn | AGAP011509 | | | 2,19466 | 0,00034 | | | | | | | | |
| G protein coupled receptor | GPRNPY3 | AGAP012378 | | | | | | | | | | | 1,5454 | 0,0000 |
| Glutaredoxin | GRX1 | AGAP011107 | 1,5252 | 0,0000 | | | 1,2772 | 0,0000 | | | -2,9794 | 0,0000 | -2,4932 | 0,0000 |
| Glutaredoxin | GRX2 | AGAP003178 | | | | | | | | | -1,9889 | 0,0000 | -2,2315 | 0,0000 |
| Glutaredoxin | GRX3 | AGAP009738 | | | | | | | | | -2,2346 | 0,0000 | -1,7484 | 0,0000 |
| Glutathione peroxidase | GPXH3 | AGAP004248 | | | | | | | | | | | -1,4907 | 0,0000 |
| Glutathione peroxidase | GPXH1 | AGAP004247 | | | | | | | | | | | | |
| Glutathione peroxidase | GPX2B | AGAP004247 | | | 1,54971 | 0 | | | | | | | | |
| Glutathione peroxidase | GPX3 | GenBank: AY745228 | | | | | 1,4856 | 0,0000 | | | 2,5722 | 0,0000 | 1,4330 | 0,0000 |
| Glutathione S-transferase | GSTD1_3 | AGAP004164 | | | | | | | | | | | -18,3029 | 0,0000 |
| Glutathione S-transferase | GSTD1_5 | AGAP004164 | -1,5627 | 0,0000 | | | | | | | -3,2898 | 0,0000 | -5,4302 | 0,0000 |
| Glutathione S-transferase | GSTD1_6 | AGAP004164 | | | | | | | | | -2,1421 | 0,0000 | -2,4233 | 0,0000 |
| Glutathione S-transferase | GSTD10 | AGAP004383 | | | | | -1,6506 | 0,0008 | | | -6,5979 | 0,0000 | -4,4537 | 0,0000 |
| Glutathione S-transferase | GSTD11 | AGAP004378 | 1,4753 | 0,0000 | | | 1,6994 | 0,0000 | | | -1,2977 | 0,0000 | -1,4938 | 0,0000 |
| Glutathione S-transferase | GSTD12 | AGAP004380 | -1,4938 | 0,0000 | | | | | | | -4,9212 | 0,0000 | -8,2535 | 0,0000 |
| Glutathione S-transferase | GSTD2 | AGAP004165 | -1,6667 | 0,0000 | | | | | | | -3,2154 | 0,0000 | -4,7076 | 0,0000 |
| Glutathione S-transferase | GSTD3 | AGAP004382 | -1,5454 | 0,0000 | 2,17046 | 0 | | | | | -3,3940 | 0,0000 | -5,0246 | 0,0000 |
| Glutathione S-transferase | GSTD4 | AGAP004381 | | | | | | | | | -4,9280 | 0,0000 | -6,0210 | 0,0000 |
| Glutathione S-transferase | GSTD5 | AGAP004173 | 8,6159 | 0,0006 | | | | | | | -14,6213 | 0,0001 | | |
| Glutathione S-transferase | GSTD6 | AGAP004379 | -1,6518 | 0,0000 | | | | | | | 1,4520 | 0,0000 | | |
| Glutathione S-transferase | GSTD7 | AGAP004163 | | | | | | | | | | | | |
| Glutathione S-transferase | GSTE1 | AGAP009195 | | | | | | | | | -2,9690 | 0,0000 | -3,4967 | 0,0000 |
| Glutathione S-transferase | GSTE2 | AGAP009194 | -1,5713 | 0,0001 | | | | | | | -3,5975 | 0,0000 | -4,7999 | 0,0000 |
| Glutathione S-transferase | GSTE3 | AGAP009197 | -1,5105 | 0,0000 | | | | | | | -1,5790 | 0,0000 | -2,7019 | 0,0000 |
| Glutathione S-transferase | GSTE4 | AGAP009193 | | | | | | | 1,3481677 | 0,00031 | -1,4928 | 0,0000 | -1,7005 | 0,0000 |
| Glutathione S-transferase | GSTE5 | AGAP009192 | | | | | | | | | -1,5465 | 0,0000 | -1,4835 | 0,0000 |
| Glutathione S-transferase | GSTE6 | AGAP009191 | | | | | | | | | | | -1,5347 | 0,0001 |
| Glutathione S-transferase | GSTE7 | AGAP009196 | -1,8391 | 0,0000 | | | | | | | -1,3947 | 0,0000 | -2,6081 | 0,0000 |
| Glutathione S-transferase | GSTE8 | AGAP009190 | 1,5746 | 0,0000 | | | | | | | -1,5199 | 0,0000 | | |
| Glutathione S-transferase | GSTO1 | AGAP005749 | 2,9019 | 0,0000 | | | 2,2176 | 0,0000 | | | | | | |
| Glutathione S-transferase | GSTMS1 | AGAP000165 | -1,4621 | 0,0000 | | | | | | | -2,9019 | 0,0000 | -5,0211 | 0,0000 |
| Glutathione S-transferase | GSTMS2 | AGAP000163 | | | | | | | | | -3,6604 | 0,0000 | -2,8699 | 0,0000 |
| Glutathione S-transferase | GSTMS3 | AGAP009946 | -1,3566 | 0,0000 | | | | | | | -2,2053 | 0,0000 | -3,1427 | 0,0000 |

| | | | Day 1 vs Day 1 | | | | Day 11 vs Day 11 | | | | Day 11 vs Day 1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | infected vs uninfected | | | | infected vs uninfected | | | | Midgut | | | |
| | | VectorBase | Midgut | | Fat Body | | Midgut | | Fat Body | | infected | | unifected | |
| **Gene description** | **Probe name** | **Gene ID** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** | **Fold** | **P-value** |
| Glutathione S-transferase | GSTS1_1 | AGAP010404 | | | | | | | | | -2,1332 | 0,0000 | -1,6586 | 0,0001 |
| Glutathione S-transferase | GSTS1_2 | GenBank: AF513639 | 2,0835 | 0,0000 | | | 1,4320 | 0,0000 | | | -2,1585 | 0,0000 | -1,4845 | 0,0000 |
| Glutathione S-transferase | GSTT1 | AGAP000761 | | | | | | | | | | | -2,4949 | 0,0000 |
| Glutathione S-transferase | GSTT2 | AGAP000888 | -1,2500 | 0,0002 | | | | | | | -1,2449 | 0,0001 | -1,5681 | 0,0000 |
| Glutathione S-transferase | GSTU1 | AGAP000947 | | | | | | | | | | | | |
| Glutathione S-transferase | GSTU2 | AGAP003257 | 1,9066 | 0,0000 | | | | | | | 1,4907 | 0,0000 | 2,4829 | 0,0000 |
| Glutathione S-transferase | GSTZ1 | AGAP002898 | | | -1,4191 | 0 | | | | | | | | |
| Midgut maltase-like protein | AGM1 | AGAP012401 | -1,5911 | 0,0000 | -1,5856 | 0 | | | | | 3,2558 | 0,0000 | 1,7484 | 0,0000 |
| NADPH P450 reductase | NADPH_P450_red | AGAP000500 | | | -1,5337 | 0,00024 | | | | | | | | |
| Nitrilase | NIT8537 | AGAP003515 | | | 2,54206 | 0 | | | | | | | | |
| Peroxidase | HPX7 | AGAP004036 | | | | | | | | | | | | |
| Peroxidase | HPX8 | AGAP004038 | | | | | | | | | | | | |
| Peroxidase | HPX5 | AGAP000051 | | | | | | | | | -1,6279 | 0,0006 | -1,9697 | 0,0000 |
| Ribosomal protein | RPL19 | AGAP004422 | -1,3660 | 0,0000 | | | | | | | 2,2361 | 0,0000 | 1,6066 | 0,0000 |
| Ribosomal protein | RPS26 | AGAP012100 | -1,5252 | 0,0000 | | | 1,2346 | 0,0000 | | | 2,4589 | 0,0000 | 1,3059 | 0,0000 |
| Salivary gland protein | GSG8 | AGAP010647 | -1,4310 | 0,0002 | | | | | | | | | -1,6155 | 0,0000 |
| Superoxide dismutase | MnSOD1 | AGAP010517 | | | | | | | | | -1,4520 | 0,0000 | -1,7569 | 0,0000 |
| Superoxide dismutase | SOD2 | AGAP005234 | -1,9752 | 0,0000 | | | | | | | | | -2,2658 | 0,0000 |
| Superoxide dismutase | SOD3A | AGAP010347 | | | | | | | | | -2,2454 | 0,0000 | -2,2847 | 0,0000 |
| Thioredoxin | TRX1 | AGAP009584 | | | | | | | | | -1,7219 | 0,0000 | -2,3182 | 0,0000 |
| Thioredoxin | TRX2 | AGAP007201 | | | | | | | | | 1,4054 | 0,0007 | 1,5401 | 0,0000 |
| Thioredoxin | TRX3 | AGAP003338 | | | | | | | | | 1,2986 | 0,0007 | | |
| Thioredoxin peroxidase | TPX1 | AGAP000396 | | | | | 1,2675 | 0,0004 | | | 1,4074 | 0,0000 | | |
| Thioredoxin peroxidase | TPX2 | AGAP011054 | | | | | 1,3407 | 0,0000 | | | | | -1,2631 | 0,0000 |
| Thioredoxin peroxidase | TPX3 | AGAP007543 | -1,4692 | 0,0000 | | | | | | | -1,4938 | 0,0000 | -2,3867 | 0,0000 |
| Thioredoxin peroxidase | TPX4 | AGAP011284 | 1,2649 | 0,0004 | | | 1,5529 | 0,0000 | 1,6483242 | 0,00011 | -4,0812 | 0,0000 | -5,0141 | 0,0000 |
| Thioredoxin peroxidase | TPX5 | AGAP007020 | | | | | | | | | | | 2,0293 | 0,0005 |
| Tubulin | TubulinA | AGAP001219 | 1,8493 | 0,0000 | | | 1,5508 | 0,0000 | | | | | | |
| Tubulin | TubulinB | AGAP010510 | 8,7604 | 0,0000 | | | 2,6117 | 0,0000 | | | -3,5578 | 0,0000 | | |
| Xanthin dehydrogenase | XD24352 | AGAP007918 | | | | | | | | | 1,8455 | 0,0000 | | |

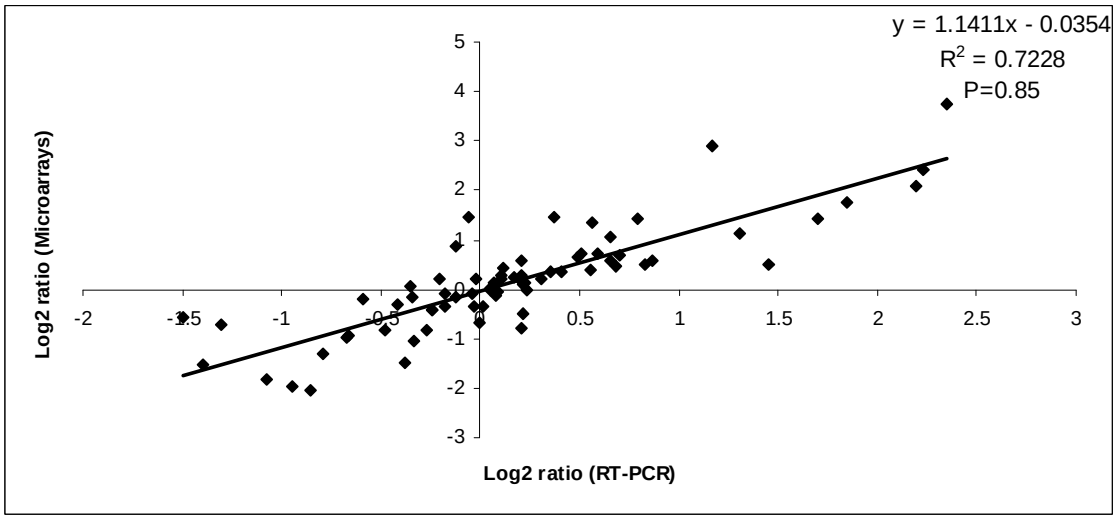
# Additional file 3

**Figure S1**. Validation of the DNA microarray analysis using quantitative RT-PCR. The mean expression values for midgut genes (A) and fat body genes (B) obtained by microarray analysis were plotted against the corresponding mean expression values obtained with quantitative RT-PCR. A high level of consistency between the two datasets was demonstrated by the Pearson correlation coefficient (P=0.884) for midgut and (P=0.85) for fat body and best-fit linear-regression analysis ($R^2$=0.7814) for midgut and ($R^2$=0.7228) for fat body.

**A) Midgut**

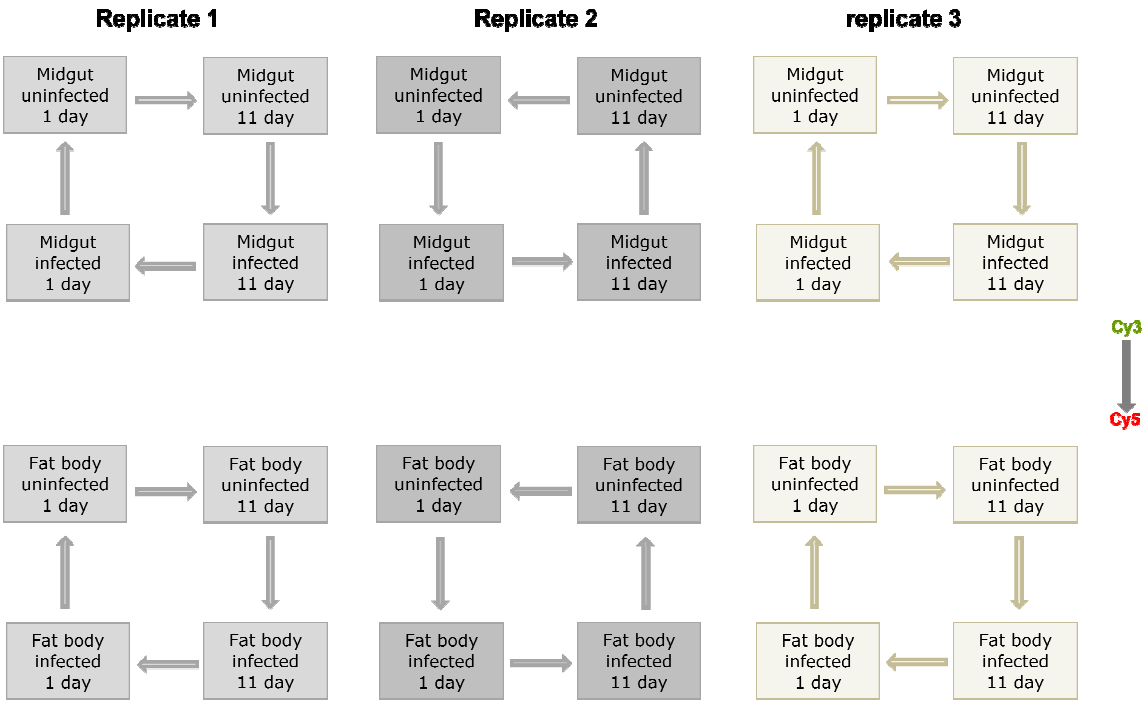



**B) Fat body**



## Additional file 4

**Figure S2**. Design of the microarray experiments. The experiments for midgut and fat body tissues followed the same layout. The boxes of the graphs represent RNA extracted from pools of 40 individuals and the arrows the microarrays to which labelled target RNA was co-hybridized. The tails of the arrows represent the samples that were labelled with a green (Cy3) and the heads those samples that were labelled with a red (Cy5) fluorescent dye. For the design matrix in *limma*, the samples from uninfected tissues collected 1 day post infection were set as the reference pool (shaded boxes). After fitting linear models the contrasts shown below the diagram were constructed for hypothesis testing of specific comparisons between RNA pools. For each of the three biological blocks (replicates 1 to 3) and factor combination a separate coefficient was included in the design matrix. The contrasts were extracted by taking the average of the three comparisons.



# Additional file 5

**Table S3**. Sequences of oligonucleotide primers used in quantitative RT-PCR validation experiments.

| Gene | Accession number | Left Sequence w/Universals (5'-3')<br>Right Sequence w/Universals (5'-3') |
|---|---|---|
| CYP6Y1 | GenBank: AF487536 | AGGTGACACTATAGAATAGAAGCAGACGCTCCAGAAGT<br>GTACGACTCACTATAGGGAATGTTTGCGCAGTGTCTCAC |
| CYP4G17 | GenBank: AY062200 | AGGTGACACTATAGAATACGATGGCTGTCATGAAAATG<br>GTACGACTCACTATAGGGACTCCTGTTCCTTCTTGACGC |
| NIT8537 | GenBank:XM_313253 | AGGTGACACTATAGAATAGCGTACGGCTATTCGTTGAT<br>GTACGACTCACTATAGGGACTTAATCTCGCCGCACATCT |
| CYP6P3 | GenBank: AF487534 | AGGTGACACTATAGAATAAGCTAATTAACGCGGTGCTG<br>GTACGACTCACTATAGGGAAAGTGTGGATTCGGAGCGTA |
| ABCC11 | TIGR: TC44749 | AGGTGACACTATAGAATATCATCTACCGGGACTTTTCG<br>GTACGACTCACTATAGGGATCCCAATGAAGCTGGATTTC |
| CYP304B1 | GenBank: AY748839 | AGGTGACACTATAGAATAGTTCAGCTGCTTTGCCAAC<br>GTACGACTCACTATAGGGAGCTGAGAATCGTGCCGTAGT |
| COEBE3C | TIGR: TC86715 | AGGTGACACTATAGAATAAGCTCATGCATCCCTTCACT<br>GTACGACTCACTATAGGGAGACGCTGGGGAATATTAGCA |
| GSTD11 | GenBank: AF513637 | AGGTGACACTATAGAATAGCTGACGAGCATCACTACCA<br>GTACGACTCACTATAGGGAGTTGATCGGGTTGAACGAGT |
| CYP6M3 | GenBank: AY193730 | AGGTGACACTATAGAATATCAAGTACCGGGTGGAGAAC<br>GTACGACTCACTATAGGGACAGCGTGGAAGATGTCTCAA |
| Tubulin B | EMBL: NAP1-P27-F-07-5 | AGGTGACACTATAGAATAGCTACCTAACAGTCGCTGCC<br>GTACGACTCACTATAGGGATTACCAATGAACGTGGACGA |
| TPX4 | GenBank: AY745235 | AGGTGACACTATAGAATAAAAAGCGCAATGTGAAGGTC<br>GTACGACTCACTATAGGGACTCCACGTTGTCCTTGTCCT |
| CYP6M2 | GenBank: AY193729 | AGGTGACACTATAGAATATTCGTCGACTCTCCTCACCT<br>GTACGACTCACTATAGGGAGAAATGTACCGGGACTGGTG |
| GSTS1-2 | GenBank: AF513639 | AGGTGACACTATAGAATACGGTGAACGATTTCCGTCTA<br>GTACGACTCACTATAGGGAATAGTCCAAAATGGCGGTGA |
| AGM1 | GenBank: X87410 | AGGTGACACTATAGAATAGTGTATCCGGACGAGGAGAA<br>GTACGACTCACTATAGGGACCGAAACTTCATTGCCAAAT |
| GSTe2 | GenBank: AF316636 | AGGTGACACTATAGAATACTGCGAAAATGTCCAACCTT<br>GTACGACTCACTATAGGGATTTGCCATACTTCGTCACCA |
| CYP4G16 | GenBank: AY062189 | AGGTGACACTATAGAATAGCCTTAGACCTTGTTGGCAG<br>GTACGACTCACTATAGGGAGCAATCAGTTTGCGATGTTG |
| CYP9L3 | GenBank: AY748831 | AGGTGACACTATAGAATAATCGGAGACACTGCGAAAGT<br>GTACGACTCACTATAGGGAATGCAATTGCGTGGTCCTAT |
| CYP9J5 | GenBank: AY748830 | AGGTGACACTATAGAATAGAAGGATGTGTTTACGCGGT<br>GTACGACTCACTATAGGGAAACCATATCGGGTCGAACAA |
| XD24352 | GenBank:AF515734 | AGGTGACACTATAGAATAAAATGATTCAGGTTGCTGCC<br>GTACGACTCACTATAGGGAATTGGTGCCAAAATCGTAGC |
| CYP12F2 | GenBank: AY176050 | AGGTGACACTATAGAATAAAATTCCAAAGGGAACGGAC<br>GTACGACTCACTATAGGGAGGATTGGCAGGAATGTTGAT |
| SP7 | GenBank: AY380336 | AGGTGACACTATAGAATACATTTCGTTGTGAACCCAAA<br>GTACGACTCACTATAGGGAAGTTCATCTCCAGCTCCAGG |

TIGR: TIGR mosquito gene index

EMBL: AnoEST database

# Chapter 4 - The Interplay Between Tubulins and P450 Cytochromes During *Plasmodium berghei* Invasion of *Anopheles gambiae* Midgut

**This chapter was published as a research paper:**

Félix RC, Silveira H (2011) The Interplay between Tubulins and P450 Cytochromes during Plasmodium berghei Invasion of Anopheles gambiae Midgut. PLoS ONE 6(8): e24181. doi:10.1371/journal.pone.0024181

# The Interplay between Tubulins and P450 Cytochromes during *Plasmodium berghei* Invasion of *Anopheles gambiae* Midgut

**Rute C. Félix*, Henrique Silveira***

UEI Parasitologia Médica, Centro de Malária e Outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal

## Abstract

***Background:*** *Plasmodium* infection increases the oxidative stress inside the mosquito, leading to a significant alteration on transcription of *Anopheles gambiae* detoxification genes. Among these detoxification genes several P450 cytochromes and tubulins were differently expressed, suggesting their involvement in the mosquito's response to parasite invasion. P450 cytochromes are usually involved in the metabolism and detoxification of several compounds, but are also regulated by several pathogens, including malaria parasite. Tubulins are extremely important as components of the cytoskeleton, which rearrangement functions as a response to malaria parasite invasion.

***Methodology/Principal Findings:*** Gene silencing methods were used to uncover the effects of *cytochrome P450 reductase*, *tubulinA* and *tubulinB* silencing on the *A. gambiae* response to *Plasmodium berghei* invasion. The role of tubulins in counter infection processes was also investigated by inhibiting their effect. Colchicine, vinblastine and paclitaxel, three different tubulin inhibitors were injected into *A. gambiae* mosquitoes. Twenty-four hours post injection these mosquitoes were infected with *P. berghei* through a blood meal from infected CD1 mice. Cytochrome P450 gene expression was measured using RT-qPCR to detect differences in cytochrome expression between silenced, inhibited and control mosquitoes. Results showed that *cytochrome P450 reductase* silencing, as well as tubulin (*A* and *B*) silencing and inhibition affected the efficiency of *Plasmodium* infection. Silencing and inhibition also affected the expression levels of cytochromes P450.

***Conclusions:*** Our results suggest the existence of a relationship between tubulins and P450 cytochromes during *A. gambiae* immune response to *P. berghei* invasion. One of the P450 cytochromes in this study, *CYP6Z2*, stands out as the potential link in this association. Further work is needed to fully understand the role of tubulin genes in the response to *Plasmodium* infection.

**Citation:** Félix RC, Silveira H (2011) The Interplay between Tubulins and P450 Cytochromes during *Plasmodium berghei* Invasion of *Anopheles gambiae* Midgut. PLoS ONE 6(8): e24181. doi:10.1371/journal.pone.0024181

**Editor:** Georges Snounou, Université Pierre et Marie Curie, France

**Received** May 9, 2011; **Accepted** August 1, 2011; **Published** August 30, 2011



**Funding:** This work was supported by a Fundacão para a Ciência e Tecnologia (www.fct.mctes.pt) grant to RCF (SFRH/BD/28024/2006) and research funds from project PTDC/SAU-MII/102596/2008. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: rfelix@ihmt.unl.pt (RCF); hsilveira@ihmt.unl.pt (HS)

## Introduction

*Plasmodium* infection starts with the ingestion of an infective blood meal. That, together with the parasite invasion of the midgut epithelium promotes an increase of the oxidative stress inside the mosquito, leading to a significant alteration on transcription of *A. gambiae* detoxification genes [1]. Among these, a high number of P450 cytochromes are differently expressed during *Plasmodium* infection, suggesting that they are involved in the mosquito response to parasite invasion.

Insect P450 cytochromes constitute a diverse superfamily of heme-containing enzymes [2] much less studied than P450 cytochromes from mammals and plants, which have well identified and characterized functions [3,4]. P450 are known to be involved in the metabolism, development and detoxification [5]. They metabolize endogenous compounds like steroids and lipids and exogenous compounds like insecticides [2,5]. In *A. gambiae* cytochromes from the *CYP6* family have been involved in metabolic resistance to insecticides [6–10]. There is also evidence that the transcription of these genes are regulated by the presence of several pathogens, including malaria parasites in the mosquito *A. gambiae* [11,12].

*Anopheles gambiae* larvae [13] and adults [1] showed P450 cytochromes to be highly enriched in the midgut and in *Drosophila melanogaster* and in *Aedes aegytpi* most P450 cytochromes were also expressed at the midgut [14,15], suggesting that gut tissue play a major role in xenobiotic detoxification and have a potential role in the protection from injurious exogenous compounds or organisms.

During *Plasmodium* infection transcription alteration of detoxification genes were associated with differential regulation of cytoskeleton genes such as *tubulinA*, *tubulinB* and *actin5C* [1]. In *A. gambiae* microtubules and hence tubulins are of great importance as endothelium cytoskeleton rearrangement may function as a key element during ookinete invasion of the midgut. [16]. A close association between tubulin/microtubules and P450 cytochromes have been described in mammals as disturbance of microtubular dynamics causes a severe impact on the cell viability and function, including the regulation of P450 cytochromes [17]. The

microtubule disarray may indirectly change the transcriptional activities of nuclear receptors which are responsible for P450 cytochromes regulation [17]. Furthermore, it was shown that colchicine, an important tubulin inhibitor, down-regulates several P450 cytochromes in human hepatocytes [18]. It was also shown that several tubulin inhibitors are metabolised by P450 cytochromes, so they are likely to induce or repress P450 gene expression [19]. Similarly in *A. gambiae*, regulation of P450 cytochrome expression might be associated with tubulins/microtubules disruption and cause suppression or induction of several P450 cytochromes during the mosquito response to parasite invasion.

The aim of this work was to clarify the role of tubulins in *A. gambiae* during the response to *Plasmodium* infection and its connection with the regulation of an important super-family of detoxification enzymes in *A. gambiae*, the P450 cytochromes.

## Materials and Methods

### Ethics Statement

The maintenance and care of experimental animals was carried out in strict accordance with the recommendations in the Europe Directive 86/609/EEC and Portuguese law (Decreto-Lei 129/92) for biomedical research involving animals. Full details of this study were approved by the Divisão Geral de Veterinária (DGV), Portugal, under Portaria 8 n°1005/92 from 23rd October. All experiments were performed under anesthesia, and all efforts were made to minimize animal suffering. All the authors directly involved with animal manipulation were licensed to conduct research using laboratory animals.

### Mosquitoes

The *A. gambiae* s.s. (molecular M form) of the Yaoundé strain mosquitoes, obtained from Instituto de Higiene e Medicina Tropical (IHMT) *A. gambiae* insectary, were used. The mosquitoes were reared at 26°C and 75% humidity on a 12/12 hour light/dark cycle. Adult mosquitoes were maintained on a 10% glucose solution until blood feeding.

### dsRNA synthesis

Primers were designed to include a T7 promoter sequence plus 20 base pairs (bp) of the sequence of the genes of interest. *Cytochrome P450 reductase* (*CPR*) (Vectorbase: AGAP000500), *tubulinA* (*tubA*) (Vectorbase: AGAP001219) and *tubulinB* (*tubB*) (Vectorbase: AGAP010510) sequences were used to amplify PCR products using *A. gambiae* genomic DNA as template. An exogenous gene, mouse *beta-2microglobulin* (*B2M*) (GenBank: NM_009735), was used to produce control dsRNA. As described above, a pair of primers that included a T7 promoter sequence plus 22 bp of *B2M* sequence were used to amplify a product using cDNA from *Mus musculus* as template. The gene-specific primers for all the genes are provided in Table S1. Each PCR product was purified using a gel extraction kit (Qiagen) and 1–2 μg of the products were used as template to synthesize dsRNA by in vitro transcription using the MEGAscript T7 kit (Ambion) following the instructions of the manufacturer. dsRNA concentration and quality were assessed by spectrometry and agarose gel.

### Silencing genes

Three day-old female mosquitoes were cold-anaesthetized and injected intrathoraxically with 69 *n*l of 3 μg $\mu l^{-1}$ solution of dsRNA (207 *n*g) for each gene of interest. In each experiment a control group was injected with dsB2M to serve as reference for intensity of infection and for quantification of gene expression levels. For double-silencing experiments the control group was injected with 138 *n*l of 3 μg $\mu l^{-1}$ of dsB2M and the test group was injected two times with 138 *n*l of a 1:1 mix with dstubA and dstubB (3 μg $\mu l^{-1}$). All the injections were performed using a microinjection system (Nanoject; Drummond Scientific). Gene silencing was confirmed 4 days after dsRNA injection by RT-qPCR using the ribosomal S7 gene (Vectorbase: AGAP010592) for normalisation. Four days after dsRNA injection, female mosquitoes were allowed to feed on *P.berghei* infected mice as described below.

### Tubulin inhibitors injection of mosquitoes

Sugar-fed two to three-day-old female mosquitoes were injected with tubulin inhibitors as described above using a microinjection system. Mosquitoes were injected with 69 *n*l of each inhibitor with final concentration being 1 μM for colchicine and 40 μM for vinblastine and paclitaxel (all inhibitors were from Sigma-Aldrich). Water was used as control for injections with colchicine and vinblastine and water with 1.7% DMSO was used as control for injection with paclitaxel. Twenty-four hours after inhibitors injection female mosquitoes were allowed to feed on *P. berghei* infected mice as described below.

### *Plasmodium berghei* infection of mosquitoes

Female CD1 mice (*Mus musculus*), obtained from the IHMT Animal facility, were intraperitoneally inoculated with $10^7$ *P. berghei* GFP CON parasitised red blood cells. The levels of parasitaemia were measured from blood samples of the mouse tail using Giemsa-stained blood films. When the parasitaemia reached 10–20% and exflagellation was observed, mice were used to infect mosquitoes. Female mosquitoes were allowed to feed directly on *P. berghei* infected mice for up to 30–45 minutes, with regular monitoring to verify that mice were anesthetised. Unfed females were removed from the cage. Fully engorged mosquitoes were kept at 19–21°C and 80% humidity for *P. berghei* development.

### Tissue collection

For mosquitoes with silenced genes, mosquito midguts were collected from pools of 30 mosquitoes 4 days after the silencing and immediately before the blood meal. Tissues were dissected from mosquitoes submerged in ice-cold DEPC treated phosphate-buffered saline (PBS) and transferred to ice-cold RNAlater (Ambion). After incubation at 4°C over night any excess RNAlater was removed and samples were stored at −20°C until RNA extraction. For all groups, mosquito midguts were collected 24 hours post-infection to determine the levels of expression of the genes in study. Eight or nine days post-infection mosquito midguts were also collected to determine infection rate (number of infected mosquitoes over total number of mosquitoes observed) and infection intensity (mean number of oocysts per infected mosquito) by fluorescence. The distribution of parasite numbers in individual mosquitoes between control and experimental groups was compared using the *Mann-Whitney* (*MW*) test. Three independent biological replicas of each experiment were performed.

### Quantitation of gene expression

Total RNA was prepared using the Nucleospin RNAII kit (Macherey-Nagel) according to the manufacturer's instructions. First strand cDNA was synthesized using oligo dT (Roche) and M-MLV Reverse Transcriptase (Promega) as described by the manufacturer. Gene expression was assessed by quantitative real-time PCR with the iQ™ SYBR® Green supermix (Bio-Rad) using the iCycler iQ™ (Bio-Rad). PCR involved an initial

denaturation at 95°C for 10 min, 40 cycles of 10 sec at 95°C and 45 sec at 62°C. Fluorescence readings were taken at 62°C after each cycle and a melting curve was obtained (60°C–99°C) to confirm the identity of the PCR product. RT-qPCR measurements were made in triplicate. For gene silencing confirmation the primers used for qPCR amplify a gene fragment non-overlapping the fragment used for dsRNA. Alongside gene silencing confirmation we also measured the levels of expression of several P450 cytochromes in order to check if the different gene silencing and the tubulin inhibition would affect the transcription these genes. Relative quantification results were normalised with the gene that codes for the ribosomal protein *S7* and analysed by the standard curve method, as optimized previously in our lab. Primers used are provided in Table S1. Three independent experiments with three replicates were performed.

### Statistical analysis

For data not normally distributed (oocyst densities) two-sample comparisons were done using a non-parametric test, the *Mann-Withney* (*MW*) test (Graphpad, Prism 5.00). The differences in the infection rate between the control group and the test groups were compared using the *Fisher's Exact test* (*F*) one-tailed (GraphPad, Prism 5.00). Comparisons of mRNA expression levels between the control groups and the test groups were done using the *Mann-Whitney* test one-tailed (GraphPad, prism 5.00).

## Results

### Effect of Silencing CPR in *P. berghei* infection

There are approximately one hundred of highly similar P450 cytochromes in the *A. gambiae* genome and their function tend to be redundant. Therefore silencing each cytochrome individually was not feasible. As an alternative to reduce the activity of P450 cytochromes, the *CPR* gene was silenced, since it is the main electron donor for P450 cytochromes.

Consistent silencing of *CPR* expression (mean = 78%, Table 1) was observed in all experiments, which allowed further analysis of the *in vivo* effects of the reduction of *CPR* activity. In all experiments dsCPR mosquitoes showed a consistent reduction in the infection rate relative to dsB2M mosquitoes, and was significant when all experiments were pooled ($p = 0{,}0391$, *Fisher's exact* test) (Table 1). When the distributions of oocysts number by infected midgut were compared between the two groups a significant reduction of *P. berghei* infection intensity was observed ($p = 0.0186$, *Mann-Whitney* test) (Figure 1A).

### Effect of tubulins silencing in *P. berghei* infection

*TubulinA* and *tubulinB* were already reported as differentially expressed during *Plasmodium* infection [1,11,13,16,20]. These tubulins are members of the microtubules that constitute the cytoskeleton. In the mosquito, the ingestion of a blood meal causes dramatic morphological changes in the cytoskeleton and their components [21]. This cytoskeleton rearrangement is seen as a robust molecular response to ookinete invasion [16]. Thus, each tubulin individually or both at the same time were silenced to determine the effect of the absence of tubulins on *Plasmodium* infection.

High levels of silenced tubulin expression were obtained both for the single silencing (*tubA* mean = 77.0% and *tubB* mean = 88.7%) and for the double silencing (*tubA* mean = 82.8% and *tubB* mean = 83.8%) (Table 1) When *tubB* was single silenced a slightly higher infection rate was observed, but this rate was essentially similar between the tubulins single or co-silenced and the dsB2M mosquitoes.

The distribution of oocysts number by infected midgut, in *tubA* and *tubB* single silencing, showed consistently higher infection intensity than the control groups, although this difference was not significant (Figure 1B). In the co-silencing, the infection intensity was similar between the dstubA/dstubB mosquitoes and the control group (Figure 1B).

**Table 1.** Effect of silencing *CPR*, *tubA*, *tubB* or co-silencing *tubA* and *tubB* and effect of injecting tubulins inhibitors on *P. berghei* infection in the mosquito.

| | N | KD (%) | Infection rate (%) | *P (Fisher's Exact test)* | Oocysts range |
|---|---|---|---|---|---|
| **Silencing** | | | | | |
| **dsB2M** | 169 | | 80.5 | | 0–178 |
| **dsCPR** | 220 | 78 | 72.3 | 0.0391* | 0.195 |
| **dsB2M** | 80 | | 71.3 | | 0–414 |
| **dsTubA** | 118 | 77 | 70.3 | 0.5100 | 0–320 |
| **dsTubB** | 112 | 88.7 | 76.8 | 0.2415 | 0–301 |
| **dsB2M/dsB2M** | 36 | | 77.8 | | 0–268 |
| **dsTubA/dsTubB** | 69 | 82.8/83.8 | 66.7 | 0.1690 | 0–161 |
| **Chemical inhibition** | | | | | |
| **Control** | 138 | n/a | 69.6 | | 0–450 |
| **Paclitaxel 40 μM** | 97 | n/a | 88.7 | 0.0004*** | 0–359 |
| **Control** | 163 | n/a | 82.2 | | 0–305 |
| **Vinblastine 40 μM** | 178 | n/a | 82.6 | 0.1214 | 0–250 |

N – total number of mosquitoes; Knock down (KD)(%) - 100*((mean expression dsB2M – mean expression dsCPR, dsTubA or dsTubB)/mean expression dsB2M); Infection rate (%) – 100*(n° of infected mosquitoes/total number of mosquitoes dissected); *Fisher's exact test* to analyse the differences in the infection rate between the control group and the test group; *indicates significant differences ($p<0.05$); *** indicates significant differences ($p<0.001$).
doi:10.1371/journal.pone.0024181.t001

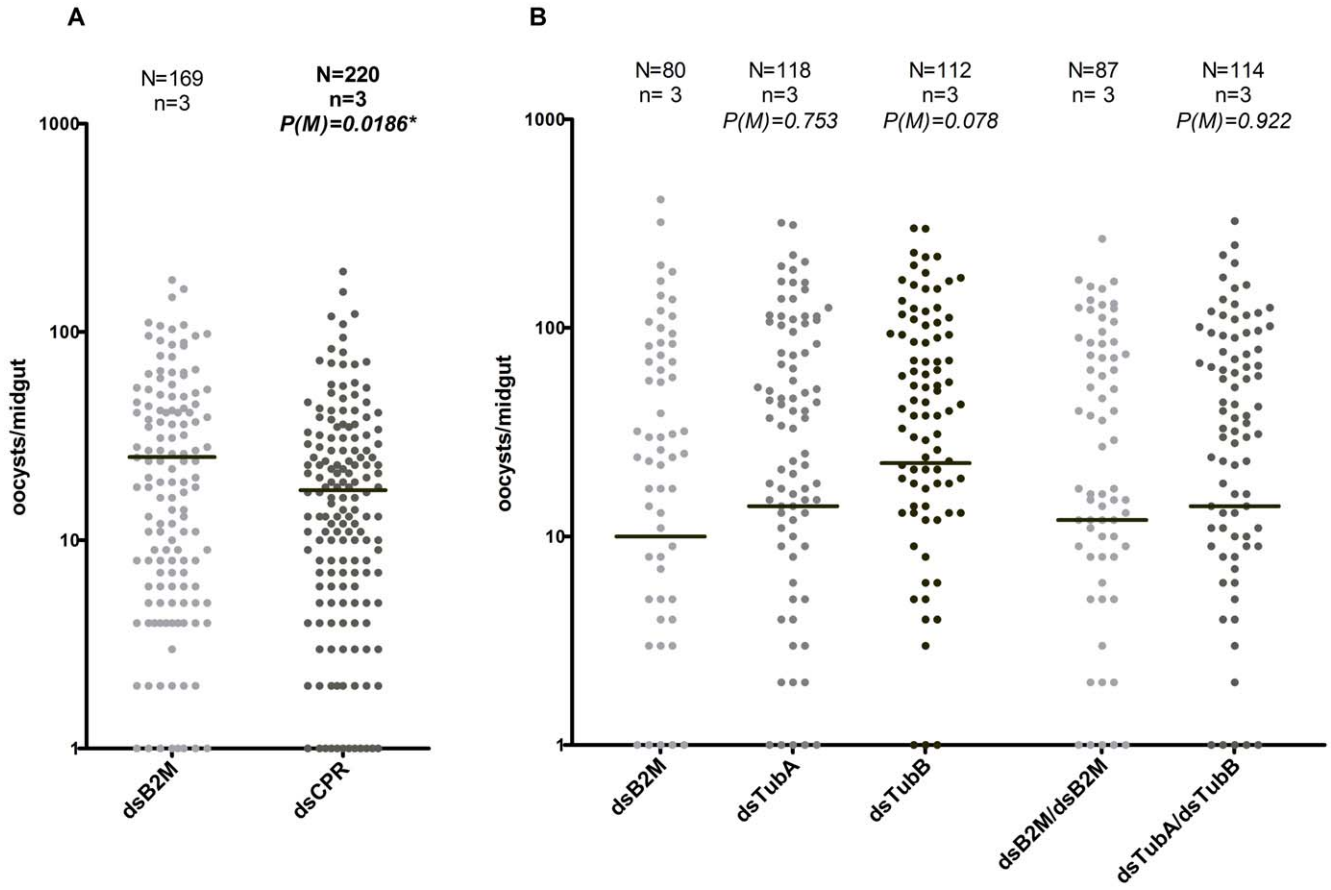



**Figure 1. Effect of silencing *CPR* (A), *tubA*, *tubB*, or co-silencing *tubA* and *tubB* (B) on *P. berghei* infection at 8 days after an infected blood meal.** The dots represent the number of parasites present on individual midguts, and the median number of oocysts is indicated by the horizontal line, where N is the total number of mosquitoes analyzed and n is the number of independent experiments. Oocysts distributions are compared using *Mann-Whitney* (M) test. * indicates significant differences ($p<0.05$).
doi:10.1371/journal.pone.0024181.g001

## Effect of tubulins inhibitors injection in *P. berghei* infection

Mosquitoes were injected with 3 tubulin inhibitors, one of each of the three major classes of tubulin inhibitors. A consistent increase in the infection rate of mosquitoes treated with paclitaxel, from the class taxoids, was observed in the 3 experiments when compared with to the control mosquitoes. This increase was highly significant ($p = 0.0004$, *Fisher's exact* test) (Table 1). The number of oocysts by infected midgut was significantly different between the control group and the group treated with paclitaxel ($p = 0.0162$, *Mann-Whitney* test) (Figure 2).

When mosquitoes were treated with vinblastine, from the class '*Vinca*' alkaloids, there were no significant differences between the control group and the test group concerning either the infection rate or the distribution of oocysts by infected midgut (Figure 2, Table 1).

When mosquitoes were injected with colchicine, from the class of colchicine binders, a high mortality was observed, although 24 h after the blood meal we were able to collect enough midguts to analyse by semi quantitative RT-PCR. The remaining mosquitoes did not survive and were all dead at day 3 after the blood meal, therefore the number of oocysts by infected midgut was not possible to determine. So, for this treatment only RT-PCR data was analysed.

## Effect of CPR silencing in P450 cytochromes expression

As *CPR* inhibition eliminates all microsomal P450 activity in the mouse model [22], the same approach was applied to evaluate whether P450 cytochromes have some role in controlling *Plasmodium* infection. The expression levels of seven P450 cytochromes, chosen among the ones reported as differentially expressed during *Plasmodium* infection [1], were analysed and differences were observed when gene expression levels were compared between the control group and the silenced *CPR* group (Figure 3). *CYP6M2* (Vectorbase: AGAP008212) and *CYP6AA1* (Vectorbase: AGAP007480) showed more pronounced differences between the control group and silenced *CPR* group, even so they were not significant (Figure 3, *Mann-Whitney test* one-tailed).

## Effect of tubulins silencing in P450 cytochromes expression

The expression of three P450 cytochromes (*CYP6M2*, *CYP6Z2* (Vectorbase: AGAP008018), and *CYP12F2* (Vectorbase: AGAP008021)), already reported as differentially expressed upon *Plasmodium* infection [1,23] and associated with insecticide resistance [7,9], was analyzed in order to detect the effect of microtubule disruption on P450 cytochromes. When *tubA* and *tubB* were silenced individually no significant differences in expression were observed. Even so, when just *tubB* was silenced, slight

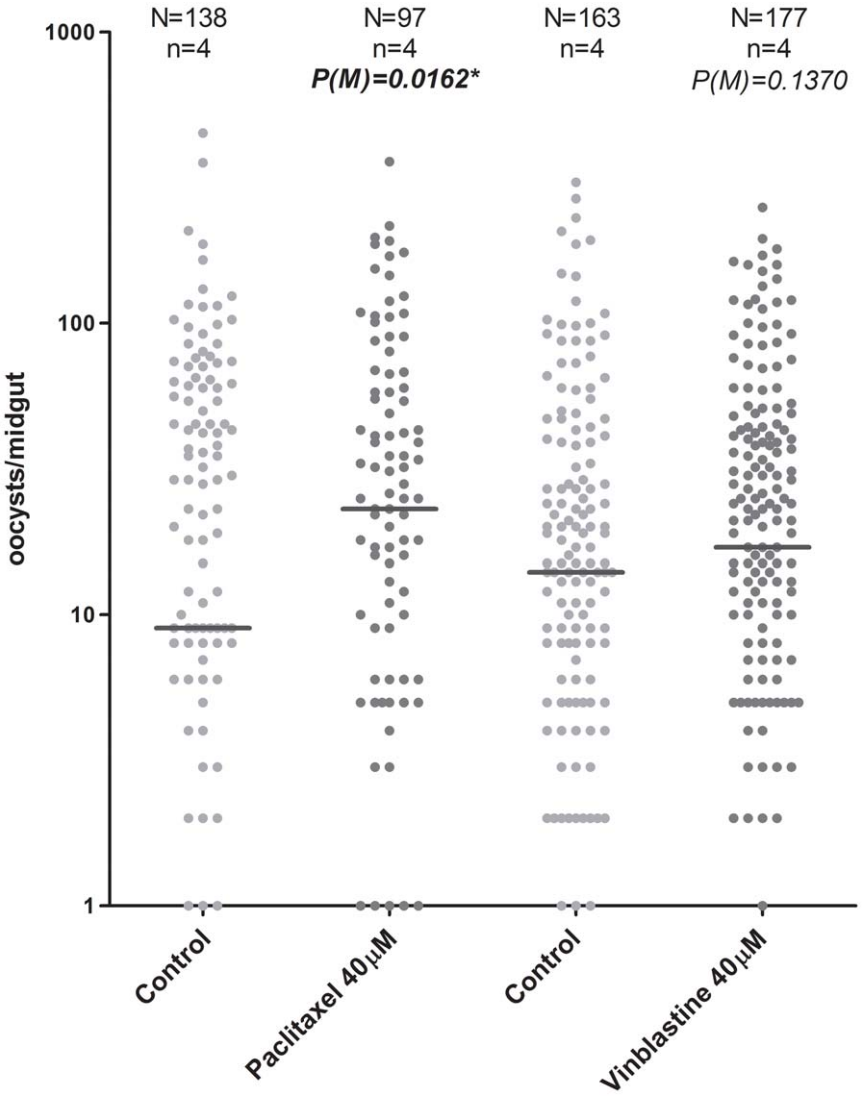



**Figure 2. Effect of tubulin inhibitors, paclitaxel and vinblastine, on *P. berghei* infection at 8 days after an infected blood meal.** The dots represent the number of parasites present on individual midguts and the median number of oocysts is represented by a horizontal line. Vinblastine 40 μM, paclitaxel 40 μM. N is the total number of mosquitoes analyzed and n is the number of independent experiments. Oocysts distributions were compared using *Mann-Whitney* (M) test. *indicates significant differences ($p<0.05$)
doi:10.1371/journal.pone.0024181.g002

decrease in expression was observed for *CYP6M2* and *CYP6Z2*, while *CYP12F2* had an opposite behavior. However, when both tubulins were KD simultaneously an increased expression was observed in all P450 cytochromes analyzed, being the difference observed in *CYP6Z2* statistically significant (Figure 4).

## Effect of tubulins inhibitors injection in P450 cytochromes expression

The effect of tubulin inhibitors (colchicine, vinblastine and paclitaxel) was analyzed on three P450 cytochromes (*CYP6M2*, *CYP6Z2* and *CYP12F2*). We observed that, as with the silencing experiments, *CYP12F2* had always a different behavior from the other two P450 cytochromes studied (Figure 5). *CYP12F2* expression levels were up-regulated after treatment with all the tubulin inhibitors, the exact opposite was observed for *CYP6M2* and *CYP6Z2*, which were down-regulated with all the tubulin inhibitors. Colchicine was the tubulin inhibitor that caused a higher response from all the genes in the study and *CYP6Z2* was the gene with the highest expression levels changes observed (Figure 5). Furthermore, *CYP6Z2* was the only P450 cytochrome where the differences between the control group and the tubulin inhibitor injected group were statistically significant (Figure 5, $p = 0.05$, *Mann-Whitney test* one-tailed) for the colchicine experiment.

## Discussion

Silencing the *CPR* gene showed that parasites become less effective in the invasion of midgut epithelium with this gene silenced, as proven by the significant reduction of the infection rate and the intensity of infection. However, the reason why this happens is still unknown. On the other hand, although being the main electron donor for P450 cytochromes activity, significant differences were not found in P450 expression profiles when *CPR*-silenced mosquitoes *versus* non-silenced ones were compared. One hypothesis is that P450 cytochromes could receive electrons from

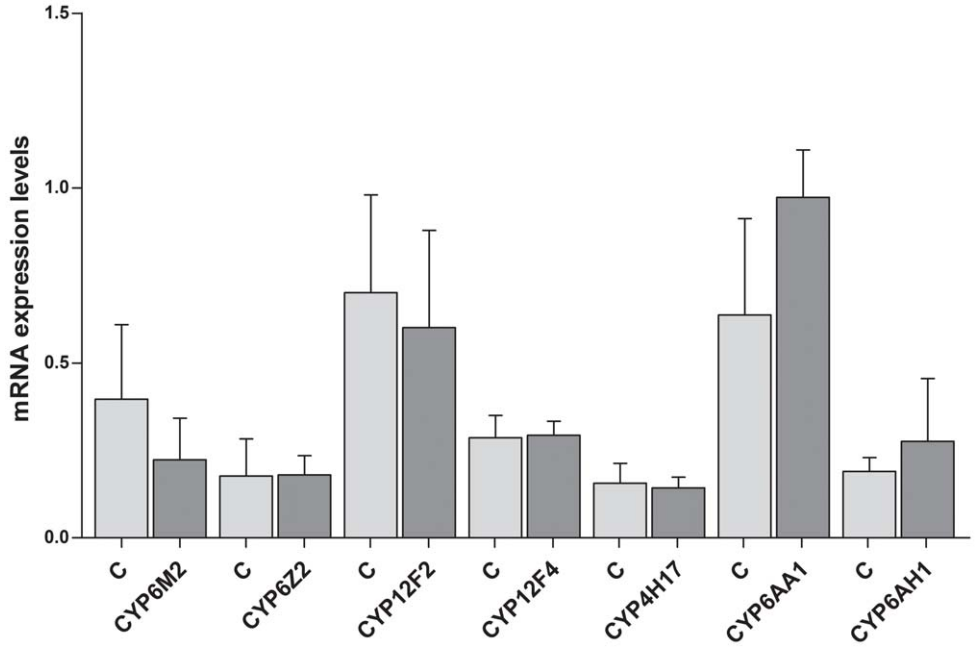



**Figure 3. P450 cytochromes mRNA expression levels in control (dsB2M-injected) and *CPR* silenced (dsCPR-injected) midguts.** Midguts were collected 24 hours after a *P. berghei* infected blood meal. Data are shown as mean ± SEM.
doi:10.1371/journal.pone.0024181.g003

other donors and that may be the reason why no differences were observed. For example, microsomal P450 cytochromes can receive electrons from cytochrome $b_5$ and cytochrome $b_5$ reductase, while P450 cytochromes in mitochondrial systems can receive electrons from an adrenodoxin-like ferredoxin coupled to an adrenodoxin reductase [2]. Also, regulation of P450 cytochrome expression depends of nuclear receptors, which may be affected by multiple mechanisms, so the silencing of the *CPR* might not have a direct impact on P450 transcription, which might explain why transcription of the P450 cytochromes studied was not affected. Knowing that *CPR* silencing did not affect P450 cytochrome expression, the reduction of *Plasmodium* infection rate and intensity observed with the silencing of this gene was not associated with P450 cytochromes being unable to perform their functions, as their expression levels were unchanged.

Tubulins are important members of microtubules that constitute the cytoskeleton. Microtubules are essential in cell division, contribute to the maintenance of cell shape and integrity and play a major role in cell motility among other important functions [24]. Their most significant characteristic is the ability to polymerize (assemble) and depolymerise (disassemble) reversibly, depending on local conditions [24]. Cytoskeleton rearrangement functions as a response to *Plasmodium* infection [16], and an association between microtubules and P450 cytochromes has been reported

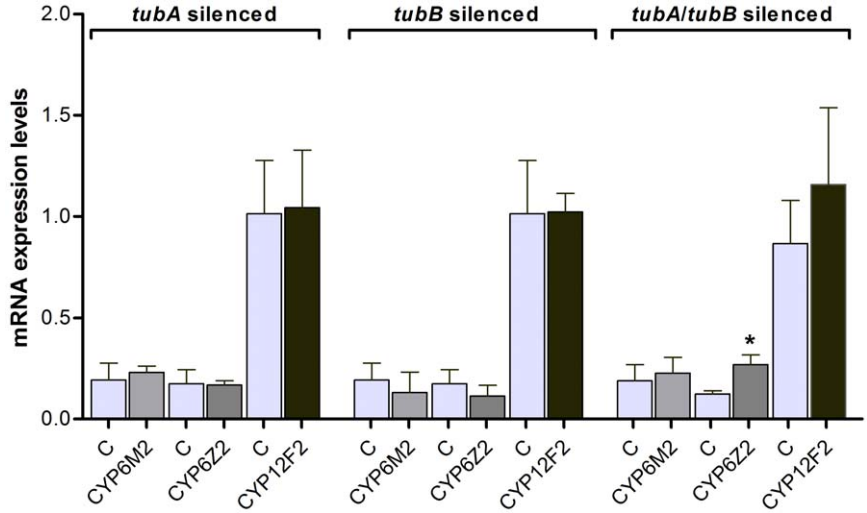



**Figure 4. Effect of silencing *tubA* or *tubB* (or co-silencing *tubA* and *tubB*) on P450 cytochromes mRNA expression levels in midguts.** Midguts were collected 24 hours after a *P. berghei* infected blood meal. Data are shown as mean ± SEM. *indicates significant differences ($p<0.05$) by *Mann-Whitney* test one-tailed.
doi:10.1371/journal.pone.0024181.g004

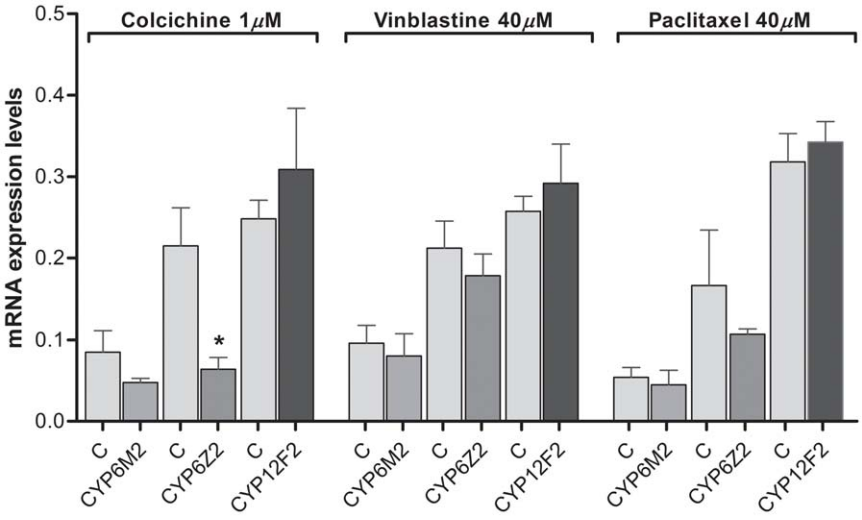



**Figure 5. Effect of tubulin inhibitors in P450 cytochromes mRNA expression levels in midguts.** Midguts were collected 24 hours after a *P. berghei* infected blood meal. Colcichine 1 μM, vinblastine 40 μM and paclitaxel 40 μM. Data are shown as mean ± SEM. *indicates significant differences ($p<0.05$) by *Mann-Whitney* test one-tailed.
doi:10.1371/journal.pone.0024181.g005

[17,18]. Both tubulin genes and many P450 cytochromes were differentially expressed during *Plasmodium* infection [1], so a connection between tubulins and P450 cytochromes in response to *Plasmodium* infection in *A. gambiae* was suggested. Silencing *tubA*, *tubB* and co-silencing *tubA*, *tubB* was performed and although some differences in the *Plasmodium* infection rate and intensity between the tested groups were observed, significant changes were not observed in infection rate neither with the single silencing nor the co-silencing. One possibility is that the cytoskeleton rearrangement is extremely complex, involving a large array of genes, and that tubulins are not crucial to the rearrangement resulting from the response to *Plasmodium*. Another possibility is that this method was not capable of truly silencing tubulins, as microtubules are dynamic polymers that are continuously being built and degraded, even if they were effectively silenced they would rapidly recover and thus mask the silencing effect. Even so, this microtubules turnover is thought to play a major role in several cellular processes requiring a change in cell shape [25], which may include the cytoskeleton rearrangement which function as a response to *Plasmodium* infection. Although not significant, oocyst density was always higher in tubulin knock down groups suggesting differences in the infection response among groups

Concerning the tubulin silencing effect in the expression of P450 cytochromes, *CYP12F2* had always a different behavior compared to the other P450 cytochromes. This may be due to the fact that these cytochromes lie at different locations: *CYP12F2* is a mitochondrial cytochrome while *CYP6M2* and *CYP6Z2* are microsomal cytochromes. In addition, they have different ways to interact with electron donors as well as different electron donors, as said above. Tubulin silencing seemed to have no effect on *CYP12F2* levels of expression, possibly because this P450 cytochrome is located in the mitochondria, thus tubulin silencing would not be able to influence genes within these organelles. An up-regulation of *CYP6M2* and *CYP6Z2* with co-silencing of *tubA* and *tubB* was observed. As said previously, microtubule disarray limits the signaling by nuclear receptors involved in P450 cytochrome regulation in mammals [17,19], consequently, the differences observed in genes expression levels may be caused by changes in nuclear receptors expression levels in response to the absence of tubulin expression.

Another approach was made to study the role of tubulins in response to *Plasmodium* infection, the injection of tubulins inhibitors. Colchicine is the inhibitor with more toxicity to mammal cells [24,26], thus it may be also very toxic to mosquitoes and that could be the reason for the high mortality of mosquitoes in these experiments. Paclitaxel injection caused a significant increased in infection rate and oocysts density in the inhibited group, while in the vinblastine injected mosquitoes there was only slightly differences between groups. The different effect of these two inhibitors in the *Plasmodium* infection must be due to the fact that these interact with microtubules via different mechanisms, while vinblastine aggregates tubulin and leads to microtubule depolymerisation, paclitaxel stabilizes microtubules by binding them to a polymer; additionally they have different binding-sites, which influence their role [24,26]. Nevertheless, these compounds may not inhibit totally tubulin, for instance, with paclitaxel microtubules can still turnover, but not to the same extent as without it [27]. As with the other inhibitors, they could just make microtubules less available, as occurs with paclitaxel [27]. On the other hand, administration of tubulin inhibitors may also be acting in the parasite tubulins in the mosquito midgut, being responsible for changes in the parasitemia levels, however, it is well known that tubulins inhibitors bind tubulins from different species with generally different affinities [24].

The down-regulation of *CYP6M2* and *CYP6Z2* expression levels with all the inhibitors was somewhat expected since microtubules-interfering agents were used, in this case colchicine, vinblastine and paclitaxel, they change the transcriptional activity of nuclear receptors responsible for the regulation of several P450 cytochromes [18,28]. Furthermore, as these inhibitors are metabolised by P450 cytochromes they could function as inducers or repressors of P450 cytochrome expression [19]. Overall, compared with tubulin silencing, it seems that tubulins inhibition had a higher effect on P450 expression levels, which suggests that different mechanisms of inhibition may affect P450 cytochromes expression in dissimilar ways. Accordingly different *CYP12F2* expression levels were obtain between the methods suggesting that they affect differently the expression of mitochondrial P450 cytochromes.

Colchicine was the inhibitor that caused higher changes in P450 expression levels, which was an expected result, as it was already

reported that colchicine down-regulated several P450 cytochromes in mammals [18]. *CYP6Z2* was the P450 cytochrome who showed significant differences in both the co-silencing of *tubA* and *tubB* and the inhibition with colchicine experiments, although in opposite directions. The reason why these two methods, with the same aim, gave such different results is not yet known, but is probably due to the different mechanism of action of the two approaches. Nevertheless, *CYP6Z2* is the most promising candidate to be directly involved with the tubulin/microtubule disarray.

In conclusion, we demonstrated that *CPR* and tubulin silencing and inhibition affected the mosquito's response to *Plasmodium*. We also showed a possible association between tubulins and P450 cytochromes in response to malaria parasite, identifying one P450 cytochrome, *CYP6Z2* as a candidate for this association. Although these silencing and inhibitions did not account for major parasite number losses during *Plasmodium* infection of the midgut they suggest that these genes may be part of a more complex response to parasite invasion. These results corroborate the importance of further studying tubulin genes to fully understand their role in the *Plasmodium* response.

## Supporting Information

**Table S1 Primers used for dsRNA synthesis and semi-quantitative real time PCR experiments and respective product length.** The underlined base pairs are the T7 promoter sequence included in the primers.
(DOC)

## Acknowledgments

We thank Ana Catarina Alves for her assistance with mosquito rearing.

## Author Contributions

Conceived and designed the experiments: RCF HS. Performed the experiments: RCF. Analyzed the data: RCF HS. Contributed reagents/materials/analysis tools: HS. Wrote the paper: RCF HS.

## References

1. Felix RC, Muller P, Ribeiro V, Ranson H, Silveira H (2010) Plasmodium infection alters Anopheles gambiae detoxification gene expression. BMC Genomics 11: 312.
2. Feyereisen R (1999) Insect P450 enzymes. Annu Rev Entomol 44: 507–533.
3. Guengerich FP (2003) Cytochromes P450, drugs, and diseases. Mol Interv 3: 194–204.
4. Schuler MA, Werck-Reichhart D (2003) Functional genomics of P450s. Annu Rev Plant Biol 54: 629–667.
5. Scott JG (1999) Cytochromes P450 and insecticide resistance. Insect Biochem Mol Biol 29: 757–777.
6. Chiu TL, Wen Z, Rupasinghe SG, Schuler MA (2008) Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT. Proc Natl Acad Sci U S A 105: 8855–8860.
7. David JP, Strode C, Vontas J, Nikou D, Vaughan A, et al. (2005) The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. Proc Natl Acad Sci U S A 102: 4080–4084.
8. Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, et al. (2008) Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. BMC Genomics 9: 538.
9. Muller P, Donnelly MJ, Ranson H (2007) Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. BMC Genomics 8: 36.
10. Nikou D, Ranson H, Hemingway J (2003) An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, Anopheles gambiae. Gene 318: 91–102.
11. Abrantes P, Dimopoulos G, Grosso AR, do Rosário V, Silveira H (2008) Chloroquine mediated modulation of Anopheles gambiae gene expression. PLoS One 3: e2587.
12. Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, et al. (2002) Genome expression analysis of Anopheles gambiae: responses to injury, bacterial challenge, and malaria infection. Proc Natl Acad Sci U S A 99: 8814–8819.
13. Neira OM, Vanekeris L, Corena-McLeod MD, Linser PJ (2008) A microarray-based analysis of transcriptional compartmentalization in the alimentary canal of Anopheles gambiae (Diptera: Culicidae) larvae. Insect Mol Biol 17: 61–72.
14. Chung H, Sztal T, Pasricha S, Sridhar M, Batterham P, et al. (2009) Characterization of Drosophila melanogaster cytochrome P450 genes. Proc Natl Acad Sci U S A 106: 5731–5736.
15. Poupardin R, Riaz MA, Vontas J, David JP, Reynaud S (2010) Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito Aedes aegypti. Insect Mol Biol 19: 185–193.
16. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC (2005) Functional genomic analysis of midgut epithelial responses in Anopheles during Plasmodium invasion. Curr Biol 15: 1185–1195.
17. Dvorak Z, Ulrichova J, Modriansky M (2005) Role of microtubules network in CYP genes expression. Curr Drug Metab 6: 545–552.
18. Dvorak Z, Modriansky M, Pichard-Garcia L, Balaguer P, Vilarem MJ, et al. (2003) Colchicine down-regulates cytochrome P450 2B6, 2C8, 2C9, and 3A4 in human hepatocytes by affecting their glucocorticoid receptor-mediated regulation. Mol Pharmacol 64: 160–169.
19. Modriansky M, Dvorak Z (2005) Microtubule disruptors and their interaction with biotransformation enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149: 213–215.
20. Marinotti O, Nguyen QK, Calvo E, James AA, Ribeiro JM (2005) Microarray analysis of genes showing variable expression following a blood meal in Anopheles gambiae. Insect Mol Biol 14: 365–373.
21. Sodja A, Fujioka H, Lemos FJ, Donnelly-Doman M, Jacobs-Lorena M (2007) Induction of actin gene expression in the mosquito midgut by blood ingestion correlates with striking changes of cell shape. J Insect Physiol 53: 833–839.
22. Henderson CJ, Otto DM, Carrie D, Magnuson MA, McLaren AW, et al. (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. J Biol Chem 278: 13480–13486.
23. Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, et al. (2006) Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS Pathog 2: e52.
24. Bell A (1998) Microtubule inhibitors as potential antimalarial agents. Parasitol Today 14: 234–240.
25. Vorobjev IA, Rodionov VI, Maly IV, Borisy GG (1999) Contribution of plus and minus end pathways to microtubule turnover. J Cell Sci 112(Pt 14): 2277–2289.
26. Fennell BJ, Naughton JA, Barlow J, Brennan G, Fairweather I, et al. (2008) Microtubules as antiparasitic drug targets. Expert Opinion on Drug Discovery 3: 501–518.
27. Stephens RE (1997) Synthesis and turnover of embryonic sea urchin ciliary proteins during selective inhibition of tubulin synthesis and assembly. Mol Biol Cell 8: 2187–2198.
28. Vrzal R, Daujat-Chavanieu M, Pascussi JM, Ulrichova J, Maurel P, et al. (2008) Microtubules-interfering agents restrict aryl hydrocarbon receptor-mediated CYP1A2 induction in primary cultures of human hepatocytes via c-jun-N-terminal kinase and glucocorticoid receptor. Eur J Pharmacol 581: 244–254.

## Supporting table 1

Primers used for dsRNA synthesis and semi-quantitative real time PCR experiments and respective product length. The underlined base pairs are the T7 promoter sequence included in the primers.

| Primer | **Vectorbase (accession number)** | **Sequence (5' to 3')** | **Product lenght(bp)** |
|---|---|---|---|
| dsCPR_Fwd | AGAP000500 | TAATACGACTCACTATAGGGAGATACTGCGGCGAGGAGAAGGAC | 380 |
| dsCPR_Rev | | TAATACGACTCACTATAGGGAGACGGAACTGGCTCTTGCGGATG | |
| dsTubA_Fwd | AGAP001219 | TAATACGACTCACTATAGGGAGACAAGGAAGATGCCGCCAAC | 432 |
| dsTubA_Rev | | TAATACGACTCACTATAGGGAGAGTGATGGACGACACAATCTGG | |
| dsTubB_Fwd | AGAP010510 | TAATACGACTCACTATAGGGAGAGGTGGAGAACACGGACGAGAC | 498 |
| dsTubB_Rev | | TAATACGACTCACTATAGGGAGACGGCGGAATATCACAGACGGC | |
| dsb2m_Fwd | GeneBank: | TAATACGACTCACTATAGGGAGACACCCCCACTGAGACTGATACA | 447 |
| dsb2m_Rev | NM_009735 | TAATACGACTCACTATAGGGAGACACCCCCACTGAGACTGATACA | |
| S7_RT_Fwd | AGAP010592 | GCCATCCTGGAGGATCTGGTA | 132 |
| S7_RT_Rev | | CGATGGTGGTCTGCTGTTCTTATCC | |
| | | | |
| CPR_RT_Fwd | AGAP000500 | CGGTGCTGGTGAAGTACGAGAC | 136 |
| CPR_RT_Rev | | CGGAACTGGCTCTTGCGGATG | |
| CYP6M2_RT_Fwd | AGAP008212 | AAGTCGGATGATGATTCGCTAACG | 169 |
| CYP6M2_RT_Rev | | GCAGGATTTCTCTCACACACTCAC | |
| CYP6Z2_RT_Fwd | AGAP008218 | CCGTTCGTCTGGTGTATTTATTTGTC | 73 |
| CYP6Z2_RT_Rev | | CAATTCAGGCTGGAGAGATGTCATG | |
| CYP12F2_RT_Fwd | AGAP008021 | GCTATGATGGAGTTGGAGATGATTAC | 118 |
| CYP12F2_RT_Rev | | GCAGCGGATTGGCAGGAATGTTG | |
| CYP12F4_RT_Fwd | AGAP008018 | CGGTTGGCAATGATGGAGATGGAG | 121 |
| CYP12F4_RT_Rev | | GATCGTTCGCAGGTATGTTGACAAG | |
| CYP4H17_RT_Fwd | AGAP008358 | TGGATCTGGTGGTGAAGGAGTC | 106 |
| CYP4H17_RT_Rev | | GCCTGCTGGAATGGTAGTGCC | |
| CYP6AA1_RT_Fwd | AGAP002862 | ACTCCACGACGGCAAGATAACG | 99 |
| CYP6AA1_RT_Rev | | TGCGGAACTGGCGGATACATAC | |
| CYP6AH1_RT_Fwd | AGAP007480 | TCTCGTCGGCCATTCGGTAACG | 122 |
| CYP6AH1_RT_Rev | | GCTCCTTCACTACACGGTCCTG | |

# Chapter 5 - Promoter analysis of three P450 cytochromes in *Anopheles gambiae*

**This chapter was submitted as a research paper:**

Felix R.C., Ribeiro V. and Silveira H. Promoter analysis of three P450 cytochromes in *Anopheles gambiae*.

## Abstract

In insects, P450 cytochromes are involved in the metabolism of numerous endogenous compounds, like juvenile hormones, ecdysteroids and fatty acids, as well as exogenous compounds like insecticides. In *Anopheles gambiae*, the major vector of malaria transmission in Sub-Saharan Africa, P450 cytochromes were described as being differently expressed in the response to microbial, parasite and insecticide challenges. However, due to the high number of P450 cytochromes it is extremely difficult to identify the exact P450 cytochrome responsible for responding to the different stimuli. A comparative analysis was performed in the promoter regions of *CYP6M2*, *CYP6Z1* and *CYP6Z2* to identify putative transcription factors binding sites and to see how these promoter regions react to different challenges. *Anopheles gambiae* Sua 5.1* cell line was transfected with reporter plasmids of each P450 cytochrome and challenged with permethrin, DDT, *Escherichia coli* and *Micrococcus luteus*, a nitric oxide donor and paclitaxel and rifampicin as controls. The work here presented showed that *CYP6M2* and *CYP6Z1* were the P450 cytochromes with greater ability to respond to different stimuli, with higher responses to insecticide exposure and bacterial challenge. On the other hand *CYP6Z2* did not respond to any of these challenges. Putative transcription factors binding sites were identified in the promoter regions of these genes.


**Keywords:** *Anopheles gambiae*; P450 cytochrome; promoter region; transcription factor binding-site

## Introduction

Cytochrome P450 proteins are one of the oldest and largest super families of enzyme proteins. These cytochromes are found in the genomes of virtually all organisms and are involved in the metabolism of a wide range of foreign chemicals of natural or synthetic origin.

In insects, P450 cytochromes (P450s) are involved in the biosynthetic pathways of ecdysteroids and juvenile hormones that are the basis for insect growth, development, feeding and reproduction (Feyereisen 1999). P450 cytochromes are also extremely important in insects because they are responsible for metabolizing endogenous and exogenous xenobiotics (Feyereisen 1999; Scott 1999). Further, several studies showed that P450 cytochromes are involved in the response to microbial and *Plasmodium* infection in *A. gambiae* (Abrantes et al., 2008; Aguilar et al., 2005; Dong et al., 2006; Felix et al., 2010). When the mosquito takes a blood meal there is an increased oxidative stress inside the midgut due to blood digestion. The oxidative stress is further increased by the presence of malaria parasites (Molina-Cruz et al., 2008). P450s are widely known for their abilities in detoxification, so they could be involved in decreasing oxidative stress inside the mosquito and, at the same time, be responsible for the elimination of parasites.

*Anopheles gambiae* P450s were also shown to be directly involved on the acquirement of insecticide resistance (David et al., 2005; Djouaka et al., 2008; Muller et al., 2007; Nikou et al., 2003). The insecticide resistance can result from the increased expression of one or more P450 cytochromes or from alterations in the primary structure of the protein, however, identifying the specific P450s enzymes which enable this response is extremely difficult (Ranson et al., 2002; Scott 1999). In *A. gambiae* genome there are 111 positively identified P450 cytochromes (Ranson et al., 2002). We focused in three specific P450 cytochromes *CYP6M2*, *CYP6Z2* and *CYP6Z1*, that have been described before has being involved in the response to microbial, parasitic and insecticide challenges in *A. gambiae* (Aguilar et al., 2005; Chiu et al., 2008; David et al., 2005; Djouaka et al., 2008; Dong et al., 2009; Felix et al., 2010; Muller et al., 2007; Pinto et al., 2009). There is no information on how these P450s perform facing these different challenges or to which compound they respond.

Studies on gene regulation through the analysis of promoter regions and the identification of putative binding sites for specific transcription factors provide insights on how the mosquito genes are activated by different challenges. Furthermore it allows the prediction of gene responses to different stimuli. Finally, it enables the association of putative binding sites and their specific transcription factors with the specific challenge they are responding to. Here we used a comparative approach to identify important transcription factor binding sites within these P450 cytochrome promoter regions and analysed their response to chemical/insecticide and microbial challenge.

## Materials and methods

### *DNA extraction*

Genomic DNA was extracted from a confluent 75 $cm^3$ flask of *A. gambiae* Sua 5.1* cells using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) following the manufacturers instructions. DNA was stored at -20 ºC.

### *Construction of reporter plasmids*

Promoter regions for constructs pGL3-CYP6M2_1 (-891/+35), pGL3-CYP6M2_2 (-456/+35), pGL3-CYP6Z1_1 (-1142/-26), pGL3-CYP6Z1_2 (-611/-26), pGL3-CYP6Z2 (-1161/+4) were amplified using forward and reverse primers that introduce 5' *Kpn*I and 3' *Xho*I restriction sites (Supplemental table 1). The PCR products were cloned into CloneJET$^{TM}$ PCR Cloning Kit (Fermentas, Thermo Fisher Scientific, Massachusetts, USA) and sequenced. Correct PCR products were double digested with *Kpn*I and *Xho*I, gel purified using Qiagen Qiaquick (Qiagen, Venlo, Netherlands) according to the manufacturers instructions. The PCR product digested and purified was then ligated into the luciferase expression vector pGL3-Basic (Promega, Wisconsin, USA) previously digested with the same enzymes. Constructs were confirmed by sequencing. The Actin 5C - *Renilla* transfection control plasmid containing the *Drosophila Actin 5C* promoter in pRL-null (Promega, Wisconsin, USA) was kindly

provided by Dr. Janet. M. Meredith. Plasmids for transfection were prepared using the NucleoBond Xtra Midi EF kit (Macherey-Nagel, Düren, Germany) following manufacturers instructions. The concentration and purity of each promoter construct was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 (Thermo Fisher Scientific, Massachusetts, USA).

### *Cell culture*

The *A. gambiae* Sua 5.1* cell line is composed of hemocyte-like cells derived from larvae of *A. gambiae* (Muller et al., 1999). Cells were maintained at 26 ºC in Schneider insect medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% heat inactivated fetal bovine serum (Lonza, Basel, Switzerland), 50 U/ml penicillin and 50 $\mu$g/ml streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA).

### *Transfection*

Transfection was mediated by FuGENE HD (Promega, Wisconsin, USA) following the manufacturers instructions. Briefly, $3x10^5$ cells in 500 $\mu$l medium were plated into individual wells of 24-well plates and grown to about 60% confluence. Schneider medium was replaced and cells were transfected using 2 $\mu$l FuGENE HD with 1 $\mu$g DNA and 5 ng Actin 5C- *Renilla* transfection control plasmid in a total volume of 30 $\mu$l antibiotic free Schneider medium. The mixture was added to the cell culture one drop at a time and cells were maintained at 26 ºC.

### *Cell challenges*

Forty-eight hours after transfection cells were stimulated with 500 $\mu$l Schneider medium supplemented with Paclitaxel, Rifampicin, bacteria, nitric oxide (NO) donor, Permethrin and Dichlorodiphenyltrichloroethane (DDT), one at a time for 24 hours.

Paclitaxel (Sigma-Aldrich, St. Louis, Missouri, USA), a mitotic inhibitor, eluted in dimethyl sulfoxide (DMSO) was diluted to a final concentration of 40 $\mu$M per well in Schneider medium. Rifampicin (Sigma-Aldrich, St. Louis, Missouri, USA), a

bactericidal antibiotic, was eluted in DMSO and then diluted to a final concentration of 10 $\mu$M per well. Schneider medium supplemented with 0.35 % DMSO was used as control for Paclitaxel and Rifampicin.

Bacteria solution was prepared by mixing heat killed *E. coli* and *M.luteus* in a 1:1 ratio in Schneider medium to a final concentration of $1x10^9$ bacteria/ $1x10^6$ cells. The NO donor, DEA-NONOate (Sigma-Aldrich, St. Louis, Missouri, USA), was eluted in Schneider medium to a final concentration of 1000 $\mu$M per well. Challenges with bacteria and NO donor used Schneider medium as control.

The insecticides permethrin and DDT (Sigma-Aldrich, St. Louis, Missouri, USA), both in methanol, were diluted in Schneider medium to a final concentration of 40 $\mu$M and 10 $\mu$M per well respectively. Schneider medium supplemented with 4 % methanol was used as control for these two insecticides.

### *Dual-luciferase reporter assays*

*Firefly* and *Renilla* luciferase activities were measured using the Luciferase assay system Dual-Glo (Promega, Wisconsin, USA). Briefly, 24 hours after the challenge cells were washed once with phosphate-buffered saline (Sigma-Aldrich, St. Louis, Missouri, USA) and then lysed in 24 wells plates with 75 $\mu$l PBS and 75 $\mu$l luciferase reagent. Ten minutes later cells were scrapped and transferred to a white 96 well plate (Nunc, Roskilde, Denmark) to measure *firefly* luciferase activity. *Renilla* activity was measured after 10 minutes incubation (minimum) with 75 $\mu$l Stop & Glo reagent. All measures were made in microplate reader Tecan Infinite® M200 (Tecan, Switzerland).

### *Statistical analysis*

Following normalization to *Renilla* activity, luciferase data from three independent experiment, with three replicates in each were pooled for analysis ($n$=9 for all except Permethrin and DDT for which $n$=8). $Log_{10}(x+1)$ transformed data, checked for normal distribution, was analysed by ANOVA (repeated measures) with Tukey's pairwise comparisons to determine the significance of differences between mean luciferase activities.

# Results

### *Identification of transcription factor binding sites*

Two different size vectors were constructed for *CYP6M2* (pGL3-Basic-CYP6M2_1 with 927 bp and pGL3-Basic-CYP6M2_2 with 456 bp) and CYP6Z1 (pGL3-Basic-CYP6Z1_1 with 1116 bp and pGL3-Basic-CYP6Z1_2 with 585 bp) to investigate the importance of putative transcription factor binding sites within the promoters. For *CYP6Z2* we only did one construct as the majority of the putative transcription factors binding sites were located far from the beginning of the gene.

The upstream regions of the P450 cytochromes were searched for insect consensus sequences matching core promoter elements using previous insect consensus described and the computer program MatInspector (Genomatix, Munich, Germany). A summary of the core promoter elements found is presented in Figure 1 and Supplemental material.

All the constructs contained a putative arthropod initiator (consensus DCAKTY, (Cherbas and Cherbas 1993)) followed by a putative TATA box (TATAAAA) 27 bp upstream for *CYP6M2*, 35 bp upstream for *CYP6Z1* and 72 bp upstream for *CYP6Z2*.

Putative nuclear factor *κ*B (NF-*κ*B) binding site with at least 80% homology to the insect consensus (consensus: GGGRNTYYY, (Kappler et al., 1993)), were identified in the promoter region of the three P450s analysed. This transcription factor is known to be induced by different agents or conditions that represent a form of stress to cells (Pahl 1999).

Several putative GATA binding sequence (consensus: WGATAR, (Ko and Engel 1993)) were identified in all the promoter regions, but none were within 12 bp of putative NF-kB binding site as reported for other insects (Kadalayil et al., 1997). GATA transcription factors (GATA) are considered positive *cis*-acting regulatory element and were reported to be involved in insect immune gene expression (Kadalayil et al., 1997).

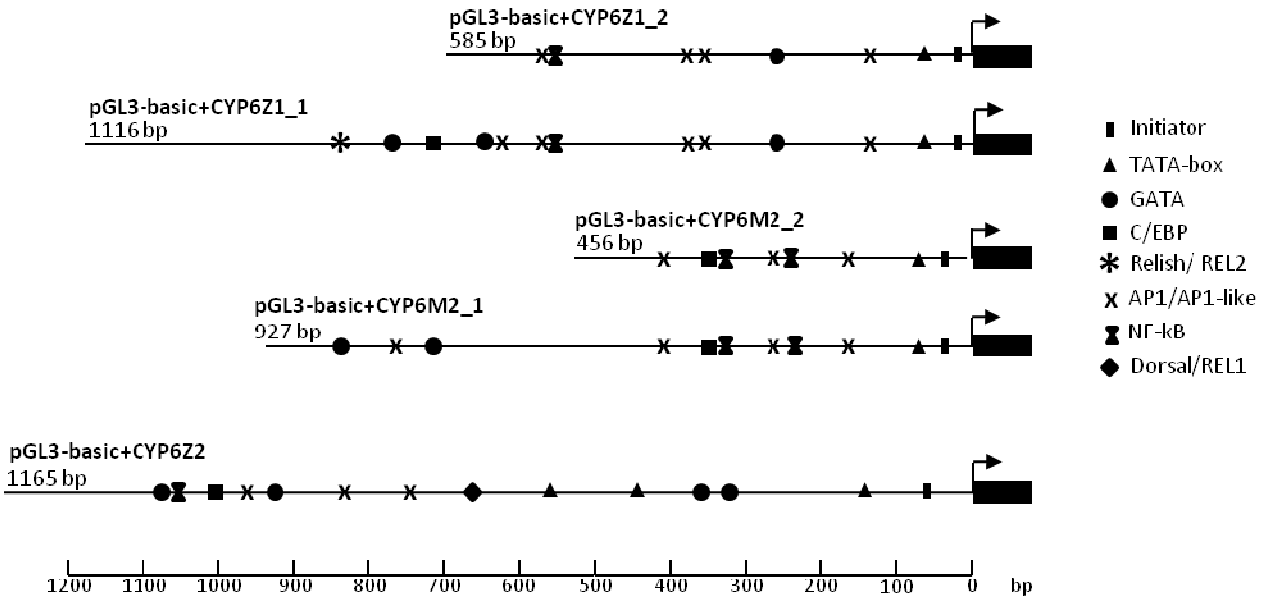



**Figure 1.** Putative transcription factor binding sites in the promoter region of *CYP6Z1*, *CYP6M2* and *CYP6Z2*.

Additionally, putative CCAAT-enhancer-binding proteins (C/EBP) binding sites (consensus: TKNNGYAAK, (Ryden and Beemon 1989)), were identified in the three promoter regions. This binding-site is necessary to transcriptional regulation and is normally closely associated with the NF-κB binding sites (Meredith et al., 2006). However here, only in the *CYP6M2* the putative C/EBP site was closely related with a putative NF-κB binding site.

Several putative activator protein-1 (AP-1) binding site (consensus: TGMSWMA, (Jayachandran and Fallon 2002)), which responds to several stimuli including stress and bacterial infections (Hess et al., 2004), were identified in all the P450s promoter regions analysed.

Binding sites for two members of the Rel/NF-kb family of transcription factors, Dorsal and Relish, implicated in the immune response in *Drosophila melanogaster*, (Senger et al., 2004), and homologues to *A. gambiae Rel 1* and *Rel 2* respectively (Christophides et

al., 2002), were identified in the promoter regions of the P450s in study. Putative dorsal binding site (consensus: GGGWWWHCB, (Senger et al., 2004)) was identified in the promoter region of *CYP6Z2* and putative relish binding sites (consensus: GGGAHNYMY, (Senger et al., 2004)) was identified in *CYP6Z1* promoter region.

***Expression of CYP6M2, CYP6Z1 and CYP6Z2 promoter regions***

All the promoter regions were cloned successfully into the pGL3-Basic vector. Compared to the promotorless vector pGL3-Basic, all the constructs were notably more active (an average of 26,7x higher for pGL3-Basic-CYP6M2_1; 55,8x for pGL3-Basic-CYP6M2_2; 16,8x for pGL3-Basic-CYP6Z1_1; 27,9x for pGL3-Basic-CYP6Z1_2 and finally 26,1x for pGL3-Basic-CYP6Z2) in dual-luciferase activities (Figs. 2-4)

***CYP6M2***

In cells stimulated with rifampicin *CYP6M2* showed no differences between the control and the stimulated cells in the longer construct, while there was a significant increase in the expression of the shorter constructed in stimulated cells. With paclitaxel, the opposite was observed (Fig. 2). A significant increase in the longer construct expression was observed for the stimulated cells, while in the shorter construct a slight decrease was observed.

DDT stimulation caused a slight increase in the expression levels of *CYP6M2* in both constructs, but in neither one this increase was significant. Meanwhile, permethrin stimulation caused a highly significant increase in the expression of the longer construct, and although the increase in expression remained in the shorter construct its significance was lost (Fig. 2).

Cell stimulation with a NO donor caused a significant increase in the expression of the *CYP6M2* longer construct (Fig. 2). No expression induction was observed with the shorter construct, suggesting that the elements responsible for this response were located in the promoter region only presented in the longer construct. Cells stimulation with bacteria, opposite to the NO, caused a significant decrease only in the shorter

construct (Fig. 2), suggesting that the different putative transcription factors binding sites present in the two constructs were used to respond to this challenge.

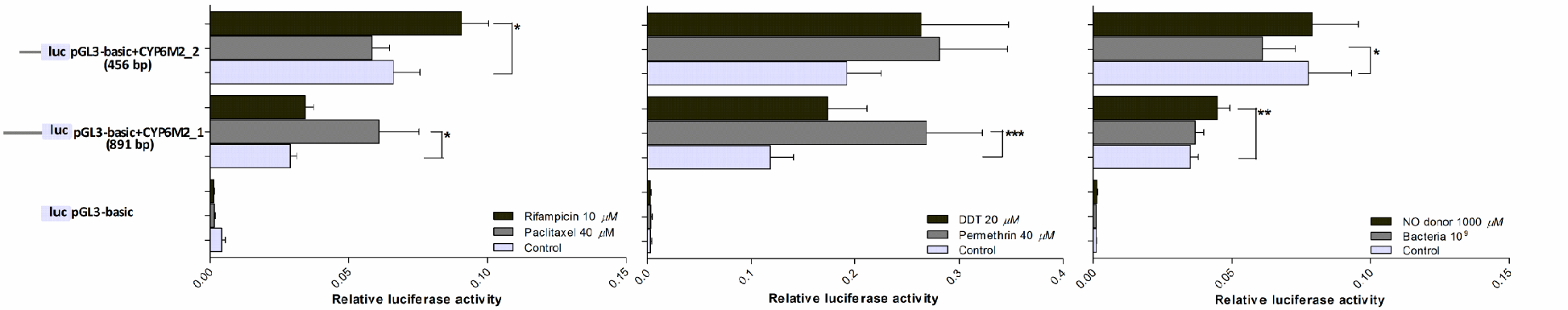



**Figure 2.** Normalised luciferase activities of *A. gambiae CYP6M2* promoter region following transfection of luciferase reporter constructs into Sua 5.1* cells. Relative luciferase activity is shown for 40 $\mu$M paclitaxel, 10 $\mu$M rifampicin and respective control; 40 $\mu$M permethrin, 20 $\mu$M DDT and respective control; $10^9$ bacteria/500 $\mu$l – mix 1:1 of *M. luteus* and *E. coli*, 1000 $\mu$M and respective control. The means and standard errors of nine replicates (eight for permetrhin and DDT stimulation), performed in three separated experiments, are shown. Significant differences between luciferase activities in control and stimulated cells are indicated with * for $P<0.05$; ** for $P<0.01$ and *** for $P<0.001$.

### *CYP6Z1*

*CYP6Z1* longer construct did not show differences in the response to the stimulation with rifampicin and paclitaxel compared to the control. On the other hand, the short construct showed a significant reduction on expression levels in cells stimulated with both compounds (Fig. 3). These results suggest that the promoter region involved in the response to these compounds is located exclusively in the region contained in the short construct.

No differences were observed in the expression levels of both *CYP6Z1* constructs in cells stimulated with DDT or permethrin compared to the control (Fig. 3), suggesting that the gene corresponding to this promoter region was not involved in the response to these insecticides.

In cells stimulated with bacteria and NO donor the expression of *CYP6Z1* (Fig. 3) showed that the longer construct did not respond to any of the compounds. In contrast, the shorter construct showed significant reduction of expression levels for cells stimulated with bacteria (Fig. 3), suggesting that the elements responsible for this response were only located in the shorter construct.

### *CYP6Z2*

The promoter region of *CYP6Z2* did not present significant differences in expression levels between the control and the entire different stimulus given (Fig. 4), suggesting that *CYP6Z2* was not involved in the response to none of these challenges or that the promoter region is located elsewhere.

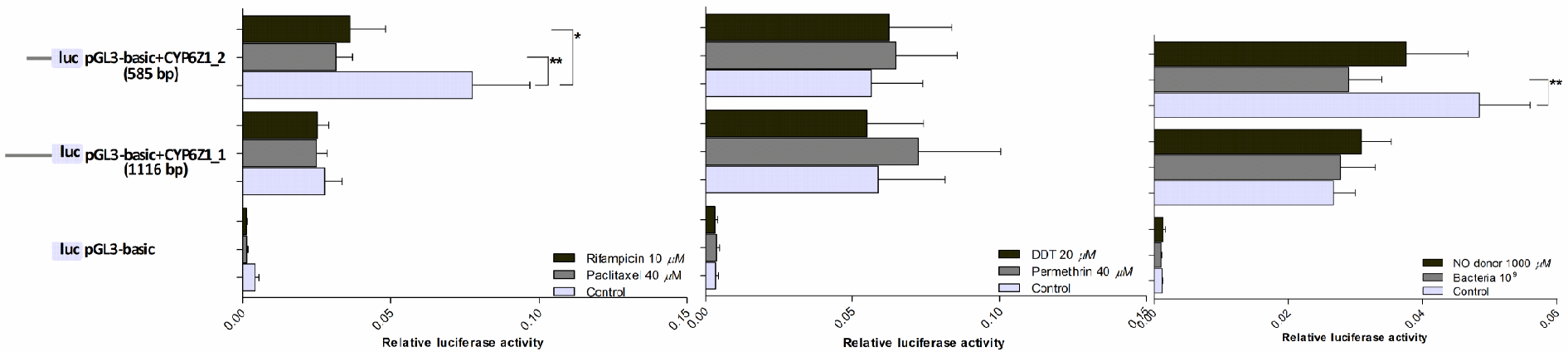



**Figure 3.** Normalised luciferase activities of *A. gambiae CYP6Z1* promoter region following transfection of luciferase reporter constructs into Sua 5.1* cells. Relative luciferase activity is shown for 40 $\mu$M paclitaxel, 10 $\mu$M rifampicin and respective control; 40 $\mu$M permethrin, 20 $\mu$M DDT and respective control; $10^9$ bacteria/500 $\mu$l – mix 1:1 of *M. luteus* and *E. coli*, 1000 $\mu$M and respective control. The means and standard errors of nine replicates (eight for permetrhin and DDT stimulation), performed in three separated experiments, are shown. Significant differences between luciferase activities in control and stimulated cells are indicated with * for $P<0.05$; and ** for $P<0.01$.

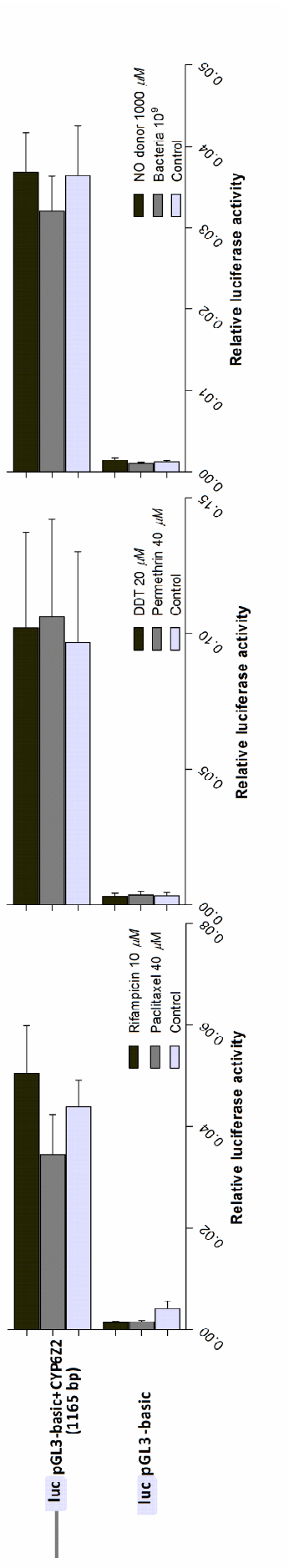



**Figure 4.** Normalised luciferase activities of *A. gambiae CYP6Z2* promoter region following transfection of luciferase reporter constructs into Sua 5.1* cells. Relative luciferase activity is shown for 40 $\mu$M paclitaxel, 10 $\mu$M rifampicin and respective control; 40 $\mu$M permethrin, 20 $\mu$M DDT and respective control; $10^9$ bacteria/500 $\mu$l – mix 1:1 of *M. luteus* and *E. coli*, 1000 $\mu$M and respective control. The means and standard errors of nine replicates (eight for permetrhin and DDT stimulation), performed in three separated experiments, are shown.

## Discussion

*CYP6Z2* had, in the promoter region, several important putative transcription factors binding-sites, but they were located so far from the start of the gene, that the long distance could be responsible for the absence of response observed here. Alternatively *CYP6Z2* may respond to other types of compounds. *CYP6Z2* could also be part of a polycistronic unit (Blumenthal 1998). *CYP6Z2* is located right after *CYP6Z1* and before *CYP6Z3*. The closeness of these genes facilitates the control of expression of several members of the same family by a common regulatory unit upstream from the first gene (*CYP6Z1*). Moreover the fact that these genes have the same orientation and similar levels of basal transcription supports this hypothesis.

*CYP6M2* was the gene that showed a greater ability to respond to different challenges. *CYP6M2* longer construct expression was significantly increased in paclitaxel stimulated cells. Paclitaxel is a mitotic inhibitor used in cancer treatment and a potential anti-parasitic drug target, that can also act as tubulin inhibitor causing a disarray in microtubules, which was shown to severely damage basal and inducible human P450 cytochromes expression (Dvorak et al., 2005; Modriansky and Dvorak 2005). Still, several studies reported paclitaxel as a strong activator of P450 expression in human cells (Ferguson et al., 2005; Harmsen et al., 2009), hence we used this compound as a control. The expression associated with the long construct of *CYP6M2* suggested that the elements responsible for this response are located in the promoter region represented in the longer construct or that the putative binding sites in the shorter construct needed upstream co-activation. The longer construct has two putative binding sites for GATA transcription factor and one putative binding site for AP-1. Together with the two putative binding sites for AP-1 and two for NF-$_K$B transcription factors present in both constructs, could be responsible for this significant increase. Furthermore, NF-$_K$B transcription factors are known to be induced by several drugs including paclitaxel (Pahl 1999).

Rifampicin is a bactericidal antibiotic, that is known to be an inducer of several P450 cytochromes (Kanebratt et al., 2008) being usually used as a positive control to P450s induction in human cells (Harmsen et al., 2009; Harmsen et al., 2010; Mani et al., 2005). In *CYP6M2* promoter region only the shorter construct showed significant

increase in the expression level, suggesting the presence of negative regulatory elements in the promoter region that was deleted. The shorter construct of *CYP6M2* has two putative NF-$_K$B transcription factors binding sites: one near a putative AP-1 binding site and the other very close to a putative binding site for C/EBP. It has been reported that the response to AP-1 was strikingly enhanced when NF-$_K$B subunits are present and vice versa (Stein et al., 1993). Later it was described by Fujioka *et al.* (2004) , that the co-activation of NF-$_K$B and AP-1 by one or several inducers can synergistically maximize the transcription of genes in response to a high diversity of stimuli (Fujioka et al., 2004). Interactions between NF-$_K$B transcription factors closely associated with C/EBP transcription factors were reported as being significant in the regulation of immune genes (Stein et al., 1993). Further, close associated NF-$_K$B and C/EBP binding sites functions as novel promoter module in mosquitoes (Meredith et al., 2006). Here the close association of NF-$_K$B with AP-1 and with C/EBP may be responsible for the increased expression of *CYP6M2*.

Paclitaxel and rifampicin also had a significant effect on the shorter construct of *CYP6Z1*, but the effect was in the opposite direction as expression was down regulated. The shorter construct had putative binding sites for GATA, AP-1 and for NF-$_K$B transcription factors. The effect of a putative NF-$_K$B binding site closely associated with a putative binding site for AP1 could contribute to this down regulation.

*CYP6M2* promoter region was also the only one to respond to the insecticide challenge. *Anopheles gambiae* P450 cytochromes have already been associated with insecticide resistance (Feyereisen 1999; Scott 1999; Scott et al., 1998). The insecticides chosen in this study, DDT and permethrin, are frequently used as a measure to prevent malaria transmission, contributing to malaria eradication. The involvement of P450 cytochromes in mosquito resistance to DDT and permethrin was already specifically reported in several studies (Chiu et al., 2008; David et al., 2005). A highly significant increase was observed for the *CYP6M2* longer construct when stimulated with permethrin. As it was described in other studies an association of these specific P450 with insecticide resistance, we were expecting a higher effect on the P450 cytochromes expression.

*CYP6M2* and *CYP6Z1* respond in the same way to heat killed bacteria stimulus. The shorter construct showed a significant reduction in expression. The most relevant putative transcription factors present in *CYP6M2* short construct were the putative NF-$_K$B binding site associated with AP-1 binding site and the NF-$_K$B binding site associated with C/EBP binding site and in the short construct of *CYP6Z1* was the putative NF-$_K$B binding site associated with AP-1 binding site. The association between these putative transcription factors binding sites may be directly involved in the response to bacterial stimulation.

An association between bacterial infection and altered P450 cytochromes expression levels in *A. gambiae* mosquitoes has been described in several studies (Aguilar et al., 2005; Baton et al., 2009; Dong et al., 2009). Studies in humans and animals showed that an inflammatory stimulus like bacterial infection down-regulates the expression and activities of P450 cytochromes in the liver (Morgan 1997; Morgan et al., 2008). Additionally, in human cells, it was showed that the use of lipopolysaccharide caused the induction of NO, that in reaction with superoxide (from P450s) generates highly reactive compounds, thus down regulation of P450 cytochromes could function as a protective mechanism for the cells (Morgan 2001). Moreover, there are studies that relate NO production and P450 cytochromes expression levels in other organisms (Khatsenko and Kikkawa 1997; Minamiyama et al., 1997; Morgan et al., 2002; Watabe et al., 2003). In our study the NO challenge caused a significant increase in the longer construct of *CYP6M2* expression. A higher response from these promoter regions was expected as it is known that the NF-$_K$B is activated by several stresses including oxidative stress (Pahl 1999) and putative binding sites for this transcription factor were found in all the P450 cytochromes in study. NO was found to play an important role in controlling the infection rate and intensity by *Plasmodium* parasites of mosquitoes (Luckhart et al., 1998). Additionally, *A. gambiae* mosquitoes showed a transcriptional activation of nitric oxide synthase, in response to bacterial and plasmodial infection (Gupta et al., 2009). While the production of NO functions as a key effector mechanism for limiting *Plasmodium* infection, in bacterial infections a decrease in NOS expression does not affect mosquito survival (Gupta et al., 2009).

In summary, we have presented here the analysis of the promoter regions of three P450 cytochromes. The most relevant putative transcription factor binding sites were NF-$_K$B,

AP-1, c/EBP, and more subtly GATA. NF-$_K$B, present in all promoter regions, may function as a central integrator of stress responses and cell survival pathways (Pahl 1999). The function of this putative binding site can be enhanced by the close presence of binding sites for C/EBP (Meredith et al., 2006), and AP-1 (Fujioka et al., 2004), which was observed here in the P450s promoter regions. The presence of several putative GATA binding sites was also observed in all the promoter regions studied. GATA transcription factor is now recognized as a positive regulatory element in several invertebrate genes (Kadalayil et al., 1997). A close association of putative GATA binding site with a putative REL binding site was described as essential for the activity of several immune genes (Senger et al., 2004), and was observed in the promoter region of *CYP6Z1*, suggesting that this gene could function as an immune related gene.

Our work confirmed that *CYP6M2* and *CYP6Z1* were involved in the response to insecticides and infection and mapped coarsely the promoter region of these P450 cytochromes indicating transcription factors that are involved in these responses. Further studies are needed to confirm the direct involvement of these putative transcription factors binding sites to the challenge response.

## Literature cited

Abrantes P, Dimopoulos G, Grosso AR, do RV, Silveira H. 2008. Chloroquine mediated modulation of Anopheles gambiae gene expression. PLoSOne 3(7):e2587.

Aguilar R, Jedlicka AE, Mintz M, Mahairaki V, Scott AL, Dimopoulos G. 2005. Global gene expression analysis of Anopheles gambiae responses to microbial challenge. Insect BiochemMolBiol 35(7):709-719.

Baton LA, Robertson A, Warr E, Strand MR, Dimopoulos G. 2009. Genome-wide transcriptomic profiling of Anopheles gambiae hemocytes reveals pathogen-specific signatures upon bacterial challenge and Plasmodium berghei infection. BMCGenomics 10:257.

Blumenthal T. 1998. Gene clusters and polycistronic transcription in eukaryotes. Bioessays 20(6):480-487.

Cherbas L, Cherbas P. 1993. The arthropod initiator: the capsite consensus plays an important role in transcription. Insect BiochemMolBiol 23(1):81-90.

Chiu TL, Wen Z, Rupasinghe SG, Schuler MA. 2008. Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT. ProcNatlAcadSciUSA 105(26):8855-8860.

Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G and others. 2002. Immunity-related genes and gene families in Anopheles gambiae. Science 298(5591):159-165.

David JP, Strode C, Vontas J, Nikou D, Vaughan A, Pignatelli PM, Louis C, Hemingway J, Ranson H. 2005. The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. ProcNatlAcadSciUSA 102(11):4080-4084.

Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, Strode C. 2008. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of

Anopheles gambiae s.s. from Southern Benin and Nigeria. BMCGenomics 9:538.

Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. 2006. Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoSPathog 2(6):e52.

Dong Y, Manfredini F, Dimopoulos G. 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoSPathog 5(5):e1000423.

Dvorak Z, Ulrichova J, Modriansky M. 2005. Role of microtubules network in CYP genes expression. CurrDrug Metab 6(6):545-552.

Felix RC, Muller P, Ribeiro V, Ranson H, Silveira H. 2010. Plasmodium infection alters Anopheles gambiae detoxification gene expression. BMCGenomics 11:312.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M, Goldstein JA. 2005. Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. MolPharmacol 68(3):747-757.

Feyereisen R. 1999. Insect P450 enzymes. AnnuRevEntomol 44:507-533.

Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T, Li Z, Evans DB, Abbruzzese JL, Chiao PJ. 2004. NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. MolCell Biol 24(17):7806-7819.

Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, Zamora RE, Barillas-Mury C. 2009. The STAT pathway mediates late-phase immunity against Plasmodium in the mosquito Anopheles gambiae. Cell HostMicrobe 5(5):498-507.

Harmsen S, Meijerman I, Beijnen JH, Schellens JH. 2009. Nuclear receptor mediated induction of cytochrome P450 3A4 by anticancer drugs: a key role for the pregnane X receptor. Cancer ChemotherPharmacol 64(1):35-43.

Harmsen S, Meijerman I, Febus CL, Maas-Bakker RF, Beijnen JH, Schellens JH. 2010. PXR-mediated induction of P-glycoprotein by anticancer drugs in a human

colon adenocarcinoma-derived cell line. Cancer ChemotherPharmacol 66(4):765-771.

Hess J, Angel P, Schorpp-Kistner M. 2004. AP-1 subunits: quarrel and harmony among siblings. JCell Sci 117(Pt 25):5965-5973.

Jayachandran G, Fallon AM. 2002. Decreased survival of mosquito cells after stable transfection with a Drosophila ecdysteroid response element: possible involvement of a 40 kDa DNA binding protein. JInsect Sci 2:21.

Kadalayil L, Petersen UM, Engstrom Y. 1997. Adjacent GATA and kappa B-like motifs regulate the expression of a Drosophila immune gene. Nucleic Acids Res 25(6):1233-1239.

Kanebratt KP, Diczfalusy U, Backstrom T, Sparve E, Bredberg E, Bottiger Y, Andersson TB, Bertilsson L. 2008. Cytochrome P450 induction by rifampicin in healthy subjects: determination using the Karolinska cocktail and the endogenous CYP3A4 marker 4beta-hydroxycholesterol. ClinPharmacolTher 84(5):589-594.

Kappler C, Meister M, Lagueux M, Gateff E, Hoffmann JA, Reichhart JM. 1993. Insect immunity. Two 17 bp repeats nesting a kappa B-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged Drosophila. EMBO J 12(4):1561-1568.

Khatsenko O, Kikkawa Y. 1997. Nitric oxide differentially affects constitutive cytochrome P450 isoforms in rat liver. JPharmacolExpTher 280(3):1463-1470.

Ko LJ, Engel JD. 1993. DNA-binding specificities of the GATA transcription factor family. MolCell Biol 13(7):4011-4022.

Luckhart S, Vodovotz Y, Cui L, Rosenberg R. 1998. The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide. ProcNatlAcadSciUSA 95(10):5700-5705.

Mani S, Huang H, Sundarababu S, Liu W, Kalpana G, Smith AB, Horwitz SB. 2005. Activation of the steroid and xenobiotic receptor (human pregnane X receptor) by nontaxane microtubule-stabilizing agents. ClinCancer Res 11(17):6359-6369.

Meredith JM, Munks RJ, Grail W, Hurd H, Eggleston P, Lehane MJ. 2006. A novel association between clustered NF-kappaB and C/EBP binding sites is required for immune regulation of mosquito Defensin genes. Insect MolBiol 15(4):393-401.

Minamiyama Y, Takemura S, Imaoka S, Funae Y, Tanimoto Y, Inoue M. 1997. Irreversible inhibition of cytochrome P450 by nitric oxide. JPharmacolExpTher 283(3):1479-1485.

Modriansky M, Dvorak Z. 2005. Microtubule disruptors and their interaction with biotransformation enzymes. BiomedPapMedFacUniv PalackyOlomoucCzechRepub 149(2):213-215.

Molina-Cruz A, DeJong RJ, Charles B, Gupta L, Kumar S, Jaramillo-Gutierrez G, Barillas-Mury C. 2008. Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium. JBiolChem 283(6):3217-3223.

Morgan ET. 1997. Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev 29(4):1129-1188.

Morgan ET. 2001. Regulation of cytochrome p450 by inflammatory mediators: why and how? Drug Metab Dispos 29(3):207-212.

Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvadi MR, Charles KA, Clarke SJ, Kacevska M, Liddle C and others. 2008. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. Drug Metab Dispos 36(2):205-216.

Morgan ET, Li-Masters T, Cheng PY. 2002. Mechanisms of cytochrome P450 regulation by inflammatory mediators. Toxicology 181-182:207-210.

Muller HM, Dimopoulos G, Blass C, Kafatos FC. 1999. A hemocyte-like cell line established from the malaria vector Anopheles gambiae expresses six prophenoloxidase genes. JBiolChem 274(17):11727-11735.

Muller P, Donnelly MJ, Ranson H. 2007. Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. BMCGenomics 8:36.

Nikou D, Ranson H, Hemingway J. 2003. An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, Anopheles gambiae. Gene 318:91-102.

Pahl HL. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18(49):6853-6866.

Pinto SB, Lombardo F, Koutsos AC, Waterhouse RM, McKay K, An C, Ramakrishnan C, Kafatos FC, Michel K. 2009. Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. ProcNatlAcadSciUSA 106(50):21270-21275.

Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, Unger MF, Collins FH, Feyereisen R. 2002. Evolution of supergene families associated with insecticide resistance. Science 298(5591):179-181.

Ryden TA, Beemon K. 1989. Avian retroviral long terminal repeats bind CCAAT/enhancer-binding protein. MolCell Biol 9(3):1155-1164.

Scott JG. 1999. Cytochromes P450 and insecticide resistance. Insect BiochemMolBiol 29(9):757-777.

Scott JG, Liu N, Wen Z. 1998. Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. Comp BiochemPhysiol CPharmacolToxicolEndocrinol 121(1-3):147-155.

Senger K, Armstrong GW, Rowell WJ, Kwan JM, Markstein M, Levine M. 2004. Immunity regulatory DNAs share common organizational features in Drosophila. MolCell 13(1):19-32.

Stein B, Baldwin AS, Jr., Ballard DW, Greene WC, Angel P, Herrlich P. 1993. Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J 12(10):3879-3891.

Watabe M, Isogai Y, Numazawa S, Yoshida T. 2003. Role of c-Myc in nitric oxide-mediated suppression of cytochrome P450 3A4. Life Sci 74(1):99-108.

## Supplemental Table 1

Primers sequences, underlined nucleotides refer to the *Kpn*I restriction site in the forward primers and *Xho*I restriction site in the reverse primers.

| Identification | Sequence 5'- 3' | Product size (pb) |
|---|---|---|
| pGL3-CYP6M2_1 Fwd | **gcagcGGTACC**ACGACCCTGAGATTAAGAG | 926 |
| pGL3-CYP6M2_2 Fwd | **gcagcGGTACC**CCTCCTCAATACCACAAGC | 491 |
| pGL3-CYP6M2 Ver | **cgataCTCGAG**CCACCAGAAATGTGAAATCC | |
| pGL3-CYP6Z1_1 Fwd | **gcagcGGTACC**GAATGAAGCGTCAAGAGTGTAA | 1116 |
| pGL3-CYP6Z1_2 Fwd | **gcagcGGTACC**CTGATTGTCAACACGATGACTC | 585 |
| pGL3-CYP6Z1 Ver | **cgataCTCGA**GGGATTTACTACGGGACACCT | |
| pGL3-CYP6Z2 Fwd | **gcagcGGTACC**AGGAAGATAAAGTTTGAG | 1169 |
| pGL3-CYP6Z2 Ver | **cgataCTCGAG**ACATTATGCCACTATGCGTT | |

# Supplemental Material

## *A) Putative transcription factors binding sites for CYP6M2 promoter region*

GAGCTAGCCGGGCTGTTGATAACTTACCCGCTCGTTTCACAGCACGTTAGCTAACAGACAGCATGAGA
GATA-binding site

GATCTTCATGCGCACACGTTTTGCGACGCATCACTGCGCAGCCGCAAGAAGGCAAGGGCGTCTAAAGG
AP-1 binding site

TAGATTGAAGCCGATGCTTGAGATAAGAAAAAATCAGTTACAGTGTTTATTATTACCTACAGATTTGAA
GATA-binding site

TCATAGAGAATAGCAGTTACATAATCAATGAAAATATGCATCTATTATCTACTTTATTTATTCTCTATGTA

TTCCATTATCAATTTTTGCTTCGAAGGCATTGCTCCTATCAATACACTCACACCCCCAAAACATAAGCTTT

TTAACTCTTCGCGCATGTTTTCACATTACTCATCACATTCCTATGTTTGATCTACGCACACCCTCTAAC**CCG**
**start shorter construct**

CCTCAACACCACAAGCTCAATTTGATACACTTCAAGATTTATGCCTATGTCATCGCTTCGCACCCCGTTCC
AP-1 binding site

CACCGTTCACTTGCACCTTCTTCTTAATCTTATCTTTTTTTGTGCAAACGACTCATAGCACCCCCCGGGAA
C/EBP binding site NF-KB binding

TACACCACGCGGACGGACTCTTCACGGTGGGATCATGCTTACTTTATTGAGTACACACGCAGGGAACTA
site AP-1 binding site NF-KB binding site

TCAGCGTCGTCGCGCACGGTGAATGAAACAGTCAACTCATCCTGACACACACGGAATTGGCGTGGCGT
AP-1 binding site

TGGCGCAAAAAAAAGGTGCGATAAGATTGGGACGAATGTGCAAAGAGAGAAGACTTGTTGTTTTGGC

TTCTAATTCGCCCTATAAAGAGAACCGGTTTGGGTGACCTCCATCATCAGTTGTCGGTGGACAGTCAAA
TATA-box Initiator

TCAATCGAACGTGGTGCTCCTCGCGTTCCAAAAA***ATG***TTTAGCTTGTTGGATTTCACTTTCTGGTGGCTC

## *B) Putative transcription factors binding sites for CYP6Z1 promoter region*

GAATGAAGCGTCAAGAGTGTAAGTATTAGGATTAACCTGTCAGTTCTAAGGATCTTGTAGGTTTGTTAT

TTATACATAAAGCAAATATATACAAGTACTGACAGCAACATATACAAGTACTAATTTAAATGACATGAA

TATGCCGCTACAATTATTTAAATAATTTGCCGTGAAATACTCCGACCATATGCTCTATAAGGGTTCTCATT

TAAGGGCTTAGGTGAAACAGAACTAAAATCCAACAATCTGTCGGTCTTGATTGACTTTAATCTACTTAC

GGGAAAACTAAATAATCACTGATTAAGACGGCAAGCTTAGACCAACTTTGGTTCTTTGATGGTGATAGA
Relish/REL2 binding site GATA-binding site

TGTAAGAACATAATGTTATACTAATCTCAAAACTAACCTGTCCTACCAACACACTTTTTGGATGCAATCA

GTTTCGGTAATAGGTCAAGGACTGCTCTTCATCAAATGTTTACATGTTGGGAAATTGATAATGTAATGC
C/EBP binding site GATA-binding site

GTGGTGTTGTCTGAGACGCAGCTGGTTCATCCCTCGGTGTA**CTG**ATTGTCAACACGATGACTCGGGAGT
AP-1 binding site **start short construct** AP-1 binding site NF-KB

TTTCCTGTTTTAAACAGTTTACATAGTCAATATTTGTTGGAAGTATAGTTTCGGAGCTCCCATTGATTGAT
binding site

ATTCTTACCAATTTCTTTTGATGAAAGCAATGCATTCAATGTCTAAGCCCCTTCTCAACAACCGAATGGG

TTAATCGTACCGACCACACCAACTTCTAAACAATCATCAACAAAACAATCAGCGATGAAAATAATGACA
AP-1 binding site

CAATGACAATCGGAAATTTCACCACCACGTTTGCGCTGCATGTAATCGTGCCGGCGGGAGAGCAGCAT
AP-1 binding site

TGCGTCCCGGTCCTGATAATCATACGGCAGGATGGGCTCGGTCGTCACGAAACCCTTGACAGTGAAAG
GATA-binding site

AGCTGTGTACGCAACGTAACCGAGCGAACCGGTTAAAACGGGAAGGAAAGAGAGACGGTATGGCTGA

TGCGTTTTTAAATATTGACACGCCACCGCATTCTATTTCTTTCTATCGCTAAGTCGCCCGCGTGTGCGGTT
AP-1 binding site

TTGTCTATAAAAGCGAGTACGTCTAGTGTTTCGCCGTCTGTTTTGCTCAGTTGCTCCATCCTAGGTGTCC
TATA-box Initiator

CGTAGTAAATCCCGAGCCTCCGTTTTGTTGCGGTCAGT***ATG***ATCCTTT

## *C) Putative transcription factors binding sites for CYP6Z2 promoter region*

CTGGCACCGAAAGGAGGCCTTCCAATGAGGATCGAGAATCGTGTTAAACATTAAGCAAAATACGCTGT
GATA

GATAATTAGGGAAATATAATACCGTTTGAAATTTATTTTTTAAGTTACTTTTAGTTACCAAAACATGAAA
binding site NF-KB binding site

TATTGCAATAAAATATACATATTAATACTCTGTATGTTCTTTTATCTTATGCCACGACATTGCACAACCTT
C/EBP binding site AP-1 binding site

TTACATTGCACAAAACCTGATAGCATCTTGAAATGTAGTTGTTCTTGCATATTAACAAGACCGCCTCCGT
GATA binding site

GGTACAGTCGTGAAATGTACGTCAGAGCCACAGGTGCGTCACACACTCGGTTTAAAGTTTAACCCATAA
AP-1 binding site

AGTGTGGAGCGCCTAAGGCAGGCTTCGACTGGAACGATTTCTTGATCAGTGCGTCACGTAATTACAAT
AP-1 binding site

AATAAAAATAGATCACTTGAATTAGAACTTCCTTGTTGTCGTTTATAATTGGTCTCTGGGTAAACGTGTA
Dorsal/REL1 binding site

CATCGGTGTGCTTGTAAATTTACAACATGCTAGGTAGGAGTTATGTTAGAGCACATATTTCATATCAAAT

TAGCTCTAATTGCTTGGCTATAAATATATCAACTGAACGATTTTTTTTTGTGATCAACTGAACATTCAAAA
TATA-box

TCAACCATATGGTCAAATAATACCTTTCTCAAATTACTACCTACATCATAATAAACGGCATTTAAATTGTG

GATTATAAATAGCAAAAACAAAAGATCGCGTGCACCCTTAAACGATACTTTTTGTTCCTACACAAACACA
TATA-box

AACACTTTGTTCTGCTCCACATGATAGCTGGAAGCTTCCAGAGTACACATTCCTATCGCTTCATGTATCA
GATA

GATAAGATGTCTAAGACATAACCCAAACAGTGCTGACCGTCACACATACAACCATACGTTTTTGATTAA
binding site

AAATATGTACGCCAGCACATACTAGCCAAAAGCACATCTTTCGCATCTAACGAACACACTTGAACCATTT

CGGAATCTCTCTCCAAAATGCTATAAAAGACTGTTGGTCTACTATGCCGATGGTTATTGTGTTTTGTGGC
TATA-box

CTCGCTCGTAAAACATAAAGAGCTTGAAGTCAGTTTTAGTTTCGTCTGCAAGTGTTTTTGGTTCATTAAT
Initiator

CTACTCTCGGCTAAACGCATAGTGGCATA***ATG***TCTCGAGATCTGCGATCTAAGTAAGCTGGCA

# Chapter 6 – General Conclusion and Future perspectives

Malaria eradication is still an unachieved goal, despite the effort put into this topic for decades. To tackle this problem, a multi-angled approach is necessary, being the control of malaria transmission one of these angles. To this end, the work presented here aims to contribute to a better understanding of the interactions between malaria parasite and its mosquito vector, focusing on the role of detoxification enzymes in response to parasite infection.

From the results presented here we can conclude that P450 cytochromes are a family of enzymes that have an important role in the response to *Plasmodium* infection by the mosquito *A. gambiae*. We saw that P450 cytochromes were some of the detoxification enzymes that had their expressions highly altered by *Plasmodium* infection together with a high number of GST enzymes, ABC transporters and genes associated with the cytoskeleton rearrangement.

This study focused on the role of cytoskeleton genes, *tubulinA* and *tubulinB*, in the response to *Plasmodium* infection and their possible association with P450 cytochromes. Nevertheless, the study of the role of GSTs in response to *Plasmodium* infection could give new and more insights on the role of detoxification enzymes in this response. A similar approach to the one used here, with reverse genetics analysis using RNA silencing, could be used to determine the role of GSTs in the parasite invasion response.

The obtained results showed that the best tissue to study the role of detoxification genes in response to parasite invasion is the midgut, as this was the most affected tissue during *Plasmodium* development.

Two main hypotheses are proposed here regarding the variation of the expression levels of detoxification enzymes during *Plasmodium* infection.

i) Increasing oxidative stress caused by the presence of the parasite is responsible for differences in the expression levels of detoxification enzymes. The mosquito response to parasite invasion includes the production of reactive oxygen species to contain the parasite, so detoxification enzyme expression could be a mechanism to eliminate or decrease oxidative stress inside the mosquito. However, further studies are still needed to clarify the association between nitric oxide and

detoxification enzymes in response to *Plasmodium* infection. A microarray-based transcriptional profiling to identify transcriptional changes in detoxification enzymes in response to a NO-donor and a NOS-inhibitor during infection, could be used to identify putative detoxification enzymes directly associated with NO in the response to *Plasmodium*.

ii) Cytoskeleton rearrangement caused by the ookinetes invasion and the oocysts burst is responsible for differences in the expression levels of detoxification enzymes. This second hypothesis seems to be supported since the cytoskeleton dynamics and remodeling were described to function as key elements in the response to ookinetes invasion of the mosquito midgut epithelium. Here we worked with *tubA* and *tubB* which are important members of microtubules. Despite our intensive work we were not able to establish a strong association between tubulins and the mosquito response to *Plasmodium* infection, as only one of the inhibitors showed significant differences in parasite intensity and infection rate. However, the results obtained strengthen the suggestion that tubulins could be part of a wider response to parasite invasion. Although this work contributed to enhance knowledge on the role of tubulins in the *A. gambiae* response to *Plasmodium* infection, the underlying mechanisms are still unknown. New studies including other genes, beside tubulins, associated with the cytoskeleton, like *actin,* would clarify the functions of these genes in the *Plasmodium* response.

Regarding the association between tubulins and P450, only one of the P450 studied, *CYP6Z2*, was shown to be a potential link of this association, and even this needs confirmation, as contradicting results were obtained with different approaches. Nevertheless, this is a promising result, as it increases the probability of other P450s being associated with tubulins in response to P*lasmodium* infection. A similar approach can be used to investigate if other P450 cytochromes are associated with tubulin alteration during infection.

This work showed that *CYP6M2* and *CYP6Z1* were involved in the response to insecticides and infection. Within the promoter regions of these P450s, several transcription factor binding sites were identified which might be involved in these responses. Site-directed mutagenesis, alone or together with electrophoretic mobility shift assays, can be used to confirm these expectations.

In conclusion, this work increased our knowledge of the role of detoxification genes and tubulins in the response to *Plasmodium* infection. A connection between tubulins and P450s during parasite infection has been unveiled. Even if the underlying mechanisms are still unknown, this work showed the importance of P450 cytochromes in the response to infection. Promising, preliminary data on how P450s recognize different challenges and how these challenges affect P450s expression levels have been generated pointing to regulatory mechanisms of transcription that need further study. Nevertheless, the interplay between the mosquito vector and the malaria parasite is extremely complex, and requires further clarification. This line of research may represent decisive help to the control of malaria transmission.